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(54) Novel glucose isomerases with an altered pH optimum.

(57) A method for selecting amino acid residues is disclosed which upon replacement will give rise to an enzyme with an altered pH optimum. The method is specific for metalloenzymes which are inactivated at low pH due to the dissociation of the metal ions. The method is based on altering the pK_a of the metal coordinating ligands or altering the K_{ass} for the metal binding. New glucose isomerases with an altered pH optimum are provided according to this method. These altered properties enable starch degradation to be performed at lower pH values.

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NOVEL GLUCOSE ISOMERASES WITH AN ALTERED PH OPTIMUM

TECHNICAL FIELD

The present invention relates to the application of protein engineering technology to improve the properties of metalloenzymes. A method for selecting amino acids which upon alteration will influence the pH-activity profile of metalloenzymes is provided. Said method is applied to glucose isomerase. The present invention also provides mutated glucose isomerase molecules with an altered pH optimum. Specifically the acidic flank of the pH-activity profile is shifted towards lower pH.

The present invention further provides recombinant glucose isomerases that advantageously can be applied in the production of fructose syrups, in particular high fructose corn syrups.

BACKGROUND OF THE INVENTION

Industrial application of glucose isomerase

In industrial starch degradation enzymes play an important role. The enzyme α-amylase is used for liquefaction of starch into dextrins with a polymerization degree of about 7-10. Subsequently the enzyme α-amyloglucosidase is used for saccharification which results in a syrup containing 92-96% glucose. The reversible isomerization of glucose intofructose is catalyzed by the enzyme glucose (or xylose) isomerase. The correct nomenclature of this enzyme is D-xylose-ketol-isomerase (EC 5.3.1.5) due to the enzyme's preference for xylose. However, because of the enzyme's major application in the conversion of glucose to fructose it is commonly called glucose isomerase. The equilibrium constant for this isomerization is close to unity so under optimal process conditions about 50% of the glucose is converted. The equilibrium mixture of glucose and fructose is known as high fructose syrup.

Fructose is much sweeter to the human taste than glucose or sucrose which makes it an economically competitive sugar substitute.

Many microorganisms which were found to produce glucose isomerase, have been applied industrially. A detailed review of the industrial use of glucose isomerases has been given by Wen-Pin Chen in Process Biochemistry, 15 June/July (1980) 3041 and August/September (1980) 36-41.

The Wen-Pin Chen reference describes culture conditions for the microorganisms, as well as recovery and purification methods for the enzyme. In addition it also summarizes the properties of glucose isomerases such as the substrate specificity, temperature optima and pH optima, heat stability and metal ion requirement.

Glucose isomerase requires a bivalent cation such as Mg^{2+}, Co^{2+}, Mn^{2+} or a combination of these cations for its catalytic activity. Determination of 3D structures of different glucose isomerases has revealed the presence of two metal ions in the monomeric unit (Farber et al., Protein Eng. 1 (1987) 459-466 ; Rey et al., Proteins 4 (1987) 165-172 ; Henrick et al., Protein Eng. 1 (1987) 467-475).

Apart from a role in the catalytic mechanism, bivalent cations are also reported to increase the thermostability of some glucose isomerases (M. Callens et al. in Enzyme Microb. Technol. 1988 (10), 695-700). Furthermore, the catalytic activity of glucose isomerase is severely inhibited by Ag^{+}, Hg^{2+}, Cu^{2+}, Zn^{2+} and Ca^{2+}.

Glucose isomerases usually have their pH optimum between 7.0 and 9.0. There are several reasons why it would be beneficial to use glucose isomerase at a lower pH value. Three of these reasons ;

a) stability of the sugar molecules,

b) adaptation both to previous and/or later process steps and

c) stability of the enzyme,

will be further described below to illustrate this.

a) Under alkaline conditions and at elevated temperatures the formation of coloured by-products and the production of a non-metabolizable sugar (D-Psicose) are a problem. The desired working pH should be around 6.0. Around this pH degradation of glucose and fructose would be minimal.

b) A lowered pH optimum is also desirable for glucose isomerase when this enzyme is to be used in combination with other enzymes, or between other enzymatic steps, for example in the manufacturing of high fructose syrups. In this process one of the other enzymatic steps, the saccharification by α-glucoamylase is performed at pH 4.5.

c) Most of the known glucose isomerases are applied at pH 7.5. This pH value is a compromise between a higher initial activity at higher pH and a better stability of the immobilized enzyme at lower pH, resulting in an optimal productivity at the pH chosen (R. v. Tilburg, Thesis : "Engineering aspects of Biocatalysts in Industrial Starch Conversion Technology", Delftse Universitaire Pers, 1983). Application of glucose isom-

erase at a pH lower than 7.5 could benefit from the longer half-life and, combined with an improved higher specific activity, would consequently increase the productivity of the immobilized enzyme at that lower pH.

From the above it can be concluded that there is need for glucose isomerases with a higher activity at lower pH values under process conditions.

Many microorganisms were screened for a glucose isomerase with a lower pH optimum. Despite many efforts, this approach did not lead to novel commercial glucose isomerases.

In order to be able to change pH-activity profile of glucose isomerases towards lower pH by protein engineering it is important to recognize the underlying effects which give rise to the rapid decrease in catalytic performance at acidic pH.

The role of metal ions in enzymes

Two different functions for metal ions in enzymes can be envisaged.

First of all metal ions can have a structural role. This means that they are involved in maintaining the proper 3D-structure and, therefore, contribute to the (thermo)stability of the enzyme molecule. An example of such a structural and stabilizing role is Ca^{2+} in the subtilisin family of serine proteinases.

Secondly, metal ions can act as a cofactor in the catalytic mechanism. In this case the enzyme activity is strictly dependent upon the presence of the metal ion in the active site. The metal ion may for instance serve as a bridge between the enzyme and the substrate (e.g. Ca^{2+} in phospholipase binds the phosphate group of the substrate) or it may activate water to become a powerful nucleophilic hydroxyl ion (Zn^{2+}-OH).

Examples are the Zn^{2+}-proteases such as thermolysin and carboxypeptidase, carbonic anhydrase (Zn^{2+}), phospholipase-A_2 (Ca^{2+}) staphylococcal nuclease (Ca^{2+}) and alkaline phosphatases (Mg^{2+}, Ca^{2+}). Examples of alpha/beta barrel enzymes which require cations to polarize a carboxyl or a carbonyl group in order to transfer hydrogen are glucose/xylose isomerase (Mg^{2+}), ribulose-1,5-biphosphate carboxylase/oxygenase (RUBISCO)(Mg^{2+}), enolase (Mg^{2+}), yeast aldolase (Mg^{2+}, K^{1+}), mandolate racemase (Mg^{2+}), muconate cycloisomerase (Mn^{2+}). In the presence of metal chelating agents (such as EDTA), these enzymes loose their activity completely.

The binding of metal ions in a protein molecule usually involves coordination by 4 or 6 ligands. Depending on the type of metal ion, different ligands are found. For instance magnesium and calcium are usually liganded by oxygen atoms from either a carbonyl group of the protein main chain, a carbonyl group from a glutamine or asparagine side chain or the carboxylate from an aspartic- or glutamic acid side chain. Zinc and copper ions are usually liganded by nitrogen atoms from a histidine side chain or the sulfur atoms from cystein and methionine.

Factors determining the pH dependence of an enzyme

The activity of an enzyme is dependent on the pH value of the aquaous medium. This dependence is caused by the (de)protonation of ionizable groups in the active site of the enzyme on the one hand, and ionizable groups of the substrate, or product (if present) on the other hand. Ionizable groups in proteins involve the side chains of the basic amino acids lysine, arginine and histidine (carrying a positive charge in the protonated form), and the acidic amino acids aspartic acid, glutamic acid, cystein and tyrosine (all carrying a negative charge upon deprotonation). Furthermore, the amino group of the N-terminus and carboxyl group of the C-terminus carry a positive and negative charge respectively. The pK_a-values of some amino acids are depicted in Table 1.

Table 1. Ionizable groups of amino acids as occurring in proteins [Cantor and Schimmel, 1980, Biophysical chemistry, W.H. Freeman, San Fransisco]

	pK_a
Positive charge (base)	
N-terminus	7.5-8.5
Lysine	10.5
Arginine	12.5
Histidine	6.0-7.0
Negative charge (acid)	
C-terminus	3.0-4.0
Aspartic acid	3.9
Glutamic acid	4.3
Cystein	8.3
Tyrosine	10.1

It should be realised that these pK_a-values are valid for model compounds and that great variations both within and between different proteins occur, due to the specific environment of the ionizable group. Electrostatic effects are known to play a fundamental role in enzyme function and structures (see J.A. Matthew et al, CRC Critical Reviews in Biochemistry, 18 (1985) 91-197). The presence of a positive charge near an ionizable group will lower its pK_a while a negative charge will cause an increase in pK_a. The magnitude of the effect decreases with the distance between the ionizable group and the charge. Moreover, the magnitude of this decrease is dependent upon the dielectric constant of the medium. Especially catalytic residues may reveal pK_a-values which deviate from these averages (see for instance Fersht, Enzyme structure and mechanism, 1985, W.H. Freeman, New York).

The pH dependence of an enzyme catalyzed reaction can be dissected into the pH-dependence of the Michaelis constant K_m and the pH-dependence of the turn-over rate constant k_{cat} (equivalent to V_{max}). These parameters represent the binding of the substrate in the ground-state and transition-state respectively. The pH-dependent (de)protonation of amino acid side chains which affect the binding of both substrate forms, or which are otherwise involved in the catalytic event (e.g. proton uptake and release as in general base catalysis), therefore, determine the pH-activity profile of an enzyme.

For instance the protonation of the histidine in the catalytic triad of serine proteases (both the trypsin- and subtilisin family) is responsible for the loss of activity at lower pH-values (<7). In this case, the pK_a of the enzyme activity is directly related to the pK_a of this histidine residue.

As a second example, the two aspartic acid residues in aspartyl proteases, such as pepsin and chymosin, can be mentioned. These groups determine the pH optimum of these proteases. The typical structural arrangement of the aspartic acids causes them to have different pK_a-values leading to the bell-shaped pH-activity profile.

It is known that altering the surface charge by extensive chemical modification can lead to significant changes in the pH dependence of catalysis. However in many cases this approach leads to inactivation and/or unwanted structural changes of the enzyme because these methods are rather unspecific. Selective chemical modification of lysines in cytochrome c was shown to have an effect on the redox-potential (D.C. Rees, J. Mol. Biol. 173, 323-326 (1980)). However, these results have been criticized because the bulky chemical reagent used for modification could perturb the structure of the protein.

Using the 3D-structure of a protein to anticipate the possibility of structural perturbation and site-directed mutagenesis, it is possible to modify the charge distribution in a protein in a very selective way.

Fersht and coworkers have shown that it is possible to manipulate the pH-activity profile of subtilisin by site-directed mutagenesis (Thomas et al, Nature, 318, 375-376 (1985) ; Russell et al, J. Mol. Biol., 193, 803-813 (1987) ; Russel and Fersht, Nature 328, 496-500 (1987)). Introduction of negatively charged groups at 10-15Å from the active site at the protein surface raises the pK_a value of the active site histidine. Conversely, making the surface more positively charged lowers the pK_a of the acidic groups. Changing either Asp99 at 13 Ångstroms

or Glu156 at 15 Ångstroms from the active site to a lysine lowers the pK_a of the active site histidine by 0.6 pH units. Changing both residues simultaneously to give a double mutant with a change of four charge units, lowers the pK_a by 1.0 pH unit. It appears that changes in Coulombic interactions can be cumulative.

Glucose isomerase mutants

WO 89/01520 (Cetus) lists a number of muteins of the xylose isomerase which may be obtained from Streptomyces rubiginosus and that may have an increased stability. The selection of possible sites that may be mutated is based on criteria differing from the ones used in the present invention. More than 300 mutants are listed but no data are presented concerning the characteristics and the alterations therein of the mutant enzyme molecules.

Methodologies for obtaining enzymes with improved properties

Enzymes with improved properties can be developed or found in several ways, for example by classical screening methods, by chemical modification of existing proteins, or by using modern genetic and protein engineering techniques.

Site-directed mutagenesis (SDM) is the most specific way of obtaining modified enzymes, enabling specific substitution of one or more amino acids by any other desired amino acid.

SUMMARY OF THE INVENTION

The subject invention provides new mutant metalloenzymes obtained by expression of genes encoding said enzymes having amino acid sequences which differ in at least one amino acid from the corresponding wildtype metalloenzymes and which exhibit altered catalytic properties. Specifically, the pH-activity profile is altered by changing the overall charge distribution around the active site.

In one of the preferred embodiments of the invention glucose isomerases are mutated.

It is another aspect of the invention to provide a method for selecting sites, in the wildtype enzyme, which can be explored by site-directed mutagenesis in order to modulate the pH-activity profile.

In still another aspect the present invention provides glucose isomerases with a more acidic pH optimum relative to the wildtype glucose isomerase.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a schematic representation of the active site of glucose isomerase from Actinoplanus missouriensis, derived from the three dimensional structure of the glucose isomerase - xylitol complex. The inhibitor is shown in full detail in the centre of the figure. For the amino acid residues only those atoms are drawn which are involved in hydrogen bonding. Amino acid residue names are in boxes drawn with solid lines, solvent molecules are in boxes drawn with dashed lines. Metal binding sites are indicated by ovals numbered 395 and 580. Dashed lines indicate electrostatic interactions : the thin dotted lines represent hydrogen bonds, the fat dashed lines the proposed ligation of the metals. Strictly conserved residues are marked by an asterix. For non-conserved residues the substitutions found are indicated.

Figure 2 shows the alignment of amino acid sequences of glucose isomerases from different sources. The complete sequence of Actinoplanes missouriensis glucose isomerase is given. The amino acid sequence of Ampullariella glucose isomerase differs from that of the published sequences (Saari, J. Bacteriol., 169, (1987) 612) by one residue : Proline 177 in the published sequence was found to be Arginine.

The Streptomyces thermovulgaris sequence has only been established up to amino acid 346. Undetermined residues are left blank. A dot indicates the absence of an amino acid residue at this position as compared to any of the other sequences. The different species are indicated by the following symbols :

Ami. : Actinoplanes missouriensis DSM 4643
Amp. : Ampullarella species ATCC31351
Svi. : Streptomyces violaceoruber LMG 7183
Smu. : Streptomyces murinus
Sth. : Streptomyces thermovulgaris DSM 40444
Art. : Arthrobacter species
Bsu. : Bacillus subtilus
Eco. : Escherichia coli
Lxy. : Lactobacillus xylosus

The secondary structure assignment was made in the structure of Actinoplanus missouriensis. The helices in the barrel are enclosed by solid lines. The β-strands are in the shaded boxes.

Figure 3 shows the pH-activity profile of glucose isomerase in the presence of 200 mM xylose and 10 mM magnesium (squares) and 1 mM manganese (circles).

Figure 4 shows the reaction scheme for the isomerisation catalyzed by glucose isomerase in the presence of metal ions.
E = enzyme, S = substrate, M = metal ion, P = product.

Figure 5 shows the pH dependence of the reaction of glucose isomerase with xylose and Mg^{2+} as observed with steady-state experiments. K_1, K_2, K_3 and K_4 are equilibrium constants explained in the text and in the reaction scheme given in Figure 4.

Figure 6 shows the pH-activity profile for the mutants K294R and K294Q in the presence of 200 mM xylose and 10 mM magnesium.

Figure 7 shows the pH-activity profiles for E186Q and E186D in the presence of 200 mM xylose and 10 mM Mg^{2+}.

Figure 8 shows the pH-activity profiles for E186D and E186Q in the presence of 200 mM xylose and 1 mM Mn^{2+}.

Figure 9 shows the pH-activity profile of the mutant D255N in the presence of 200 mM xylose and 1 mM manganese.

Figures 10-20 show the normalized pH-activity profiles for the following mutants :
F254K (Fig.10), F94R (Fig.11), F61K (Fig.12), A25K (Fig.13), D57N (Fig.14), L258K (Fig.15), Q204K (Fig.16), R23Q (Fig.17), H54N (Fig.18), H290N (Fig.19), T95D (Fig.20).
Conditions are mentioned in the Figures.

Figure 21 shows the normalized pH-activity profile for mutant F61KK253R. Conditions are mentioned in the Figure.

DETAILED DESCRIPTION OF THE INVENTION

The present invention describes the modification of enzymes to improve their industrial applicability. The invention makes use of recombinant DNA techniques. Such techniques provide a strong tool for obtaining desired amino acid replacements in the protein of choice. Because of the virtually unlimited amount of possible amino acid replacements it is preferable to use a selective approach. The present approach relies on the well coordinated application of protein crystallography, molecular modelling and computational methods, enzymology and kinetics, molecular biology and protein chemistry techniques. The strategies for the identification of targeted mutations are innovative in the sense that it is recognized that point mutations rarely cause only local perturbations. Mutations generally affect several different properties of the protein at once. Therefore, although the disclosed strategies make use of well established structure-function relationships, they also provide a rational way to avoid or correct unwanted alterations of secondary properties.

Extensive biochemical investigation of the designed mutants results in the identification of mutants with improved properties.

By 'improved properties' as used herein in connection with the present glucose isomerase enzymes we mean enzymes in which the acidic flank of the pH-activity profile shifted towards a more acidic pH optimum relative to the corresponding wildtype enzymes.

It was established that the pH-activity profile of wildtype glucose isomerase reveals a decrease in activity at both acidic pH below 7.0 and at alkaline pH beyond pH 8.0 (Example 1 - Figure 3). As discussed earlier it would be preferable to use glucose isomerase at lower pH.

Surprisingly, it was found that the drop in activity at the acidic side of the pH-activity profile is caused by the protonation of one or more amino acid side chains, which are directly involved in the coordination of the catalytic metal ion of glucose isomerase. This was deduced from the fact that the apparent association constants for metal binding, as determined by steady-state kinetics, showed a similar pH-dependency (Example 1 - Figure 5). This can be described by the following model :

$$\begin{array}{ccc} \text{site} & + & Mg^{2+} \underset{}{\overset{K_{ass}}{\rightleftharpoons}} \text{site:}Mg^{2+} \\ pK_a \;\Big\| & & \\ \text{site:}H^+ & & \end{array}$$

In which "site" refers to the geometrical binding site composed of the different (ionizable) ligands. The

enzyme is only active when the site is occupied by an Mg^{2+}-ion, which, in turn, can only bind to the unprotonated metal binding site ("site") and not the protonated one ("site :H^+"). The pH-dependent protonation of the metal binding site is characterized by the pK_a, whereas the metal binding to the unprotonated site is characterized by the association constant K_{ass}. The apparent association constant for metal binding is a function of the true K_{ass}, the pK_a and the pH and can be described as follows :

$$K_{ass}^{apparent} = K_{ass} * (1 + 10^{(pK_a - pH)})^{-1}$$

The rate of the reaction is proportional to the fraction of the enzyme molecules which is complexed with Mg^{2+}. This fraction increases at a higher $[Mg^{2+}]*K_{ass}^{apparent}$.

Although the presented model was described after an observation made in glucose isomerase, it is obvious that this model, and the method derived therefrom, can be used for metalloenzymes in general, provided that the inactivation at low pH is due to the dissociation of the metal ions.

The drop in activity observed at alkaline pH is due to a decrease in the maximal velocity (V_{max}), reflecting the deprotonation of an amino acid residue that is essential for the catalytic mechanism.

From the model described above, which can be used to explain the decrease in activity at the acidic side of the pH-activity profile, it can be deduced that in order to increase the activity of glucose isomerase at lower pH values, K_{ass} has to be increased and/or the pK_a has to be decreased.

Therefore, in one embodiment of the invention, DNA sequences coding for metalloenzymes such as glucose isomerases are mutated in such a way that the mutant proteins reveal a change in the pH-activity profile as a result of a change in pK_a of amino acid side chains acting as ligands in the metal binding.

Shift of the pH-activity profile of metalloenzymes to lower pH, by changing the pK_a of amino acid side chains

In order to shift the pK_a's of metal coordinating ligands to a more acidic pH, residues have to be introduced which increase the overall positive charge around the metal binding site of metalloenzymes. Consequently, the pH dependence of the activity of metalloenzymes, for which the activity at the acidic side of the pH optimum is caused by the pK_a of metal binding, will change accordingly. This charge alteration will stabilize the negative charge of the ionizable groups which are responsible for the pH dependence of the metal binding through long range electrostatic effects.

According to a preferred embodiment the shift of the pH-activity profile of glucose isomerase to a more acidic pH is achieved by increasing the overall positive charge around the active site of glucose isomerase.

Around neutral pH a net increase in positive charge can be obtained by :

- replacing a negatively charged residue (Asp or Glu) by a neutral one
- replacing a neutral residue by a positively charged one (Arg or Lys)
- replacing a negatively charged residue by a positively one.

For the selection of residues, which are suitable to be mutated, the following criteria can be formulated :

I. Select those positions at which substitution will lead to a net increase in positive charge within a 15 Ångstroms radius around the target residues. The target residues are the ionizable groups which are involved in the coordination of the cation.

Eliminate from this collection :

- All positions that already contain a positive charge : arginine or lysine.
- All positions that cannot harbour an arginine or a lysine because these residues would lead to inadmissible Van der Waals overlap with the backbone atoms of the protein.
- All positions at which an arginine or a lysine would need extensive adaptation of additional positions in the direct environment in order to avoid Van der Waals overlap.
- All positions at which substitution into arginine or lysine would lead to a buried uncompensated charge in a hydrofobic cluster.
- All positions at which residues should not be replaced because they are involved in typical structural arrangements such as : salt bridges, packing of helices, stabilization of helices by keeping a negative charge at the start of a helix, initiation of helices, e.g. prolines at the start of a helix, Phi-psi angles which are outside the allowed region for the residue that is going to be inserted.

II. In a preferred embodiment the following amino acids are also eliminated from the collection :

- All residues that are implied in catalysis, cofactor binding (such a metal ions and nucleotides)
- All positions that appear to be strictly conserved among homologous enzymes (if available).

III. Subsequently a priority can be attributed to each possible mutation site. This is done by inspection of the structural environment of the residue, the distance to the 'target' residues, the hydrogen bonding pattern in which the residue at said site is involved and the solvent accessibility.

In order to avoid masking of the electrostatic interactions by counter-ions, introduction of charges at sites which can not be shielded from the target residues by solvent, are to prefer. So in general, due to the dif-

ference in dielectric properties between the protein and the solvent, charges, which cannot be solvated completely due to the fact that they are buried in the interior of the protein or partially buried in clefts on the protein surface, are more likely to cause effects than charges that are completely solvated. Moreover, in the case of glucose isomerase, the conversion of glucose into fructose is performed at low ionic strength, and therefore, shielding by counter-ions is expected to interfere less seriously with the newly designed charge-charge interaction in the novel glucose isomerase described within the embodiment of the invention.

Criteria for the assignment of low priority to the above selected sites, when replacing into a positive charge are :

- The introduction of a positive charge and/or elimination of a negative charge will affect the integrity of the quaternary structure.
- The site is completely solvent accessible so that an introduced charge is expected to be shielded from the target residues by the solvent, which therefore will diminish the effect on pKa of the target residues.

Likewise, increasing the overall negative charge around the metal binding site will shift the pK_a's to more basic pH-values.

Shift of the pH-activity profile of metalloenzymes to lower pH, by increasing K_{ass} for the metal binding

In another preferred embodiment of the subject invention the shift of the pH-activity profile to a lower pH is achieved by increasing the K_{ass}.

The shift of the pH-activity profile of metalloenzymes to a more acidic pH can be achieved by increasing the association constant for metal binding. The association constant for metal binding can be increased by optimization of the coordination of the metals by the ligands. This may be realised by the introduction of better ligands or by introducing more ligands. Electrostatic interactions can contribute to the association constant for metal binding over much longer distances.

In another preferred embodiment the acidic flank of the pH-activity profile of glucose isomerase is shifted to lower pH by increasing the association constant for metal binding.

Glucose isomerase binds two magnesium ions per subunit, resulting in the binding of eight cations per tetramer, thereby increasing the total charge by +16 (see Figure 1). Both binding sites are located at the C-terminal end of the β-barrel. The 'Down' binding sites is located rather deep in the barrel and, in the xylitol complex, directly binds two oxygens from the inhibitor. The second binding site ('Up') is located near the end of the β-barrel, close to the active site cleft and the subunit interface.

In general, when a positively charged ion binds to a second particle, the association and dissociation rate constants as well as the overall equilibrium affinity constant will depend upon the charge of the second particle. Repulsion occurs when the particle is also positively charged and attraction occurs between opposite charges. For small ions, and in certain cases also proteins, this effect can be quantified by studying the ionic strength dependence of the reaction rate. The rate of association of oppositely charged ions will decrease with increasing ionic strength, the rate of association of the same charges will increase with increasing ionic strength, and when one of the particles is not charged there is no effect of the ionic strenght.

The affinity of glucose isomerase for magnesium decreases with increasing ionic strength which is consistent with an overall negative charge of the glucose isomerase binding site. The binding of the cation may be altered by the introduction of a charged amino acids at the protein surface along the trajectory of the cation upon entrance of the active site. More specifically, this invention relates to the use of electrostatic forces to alter the association rate constant of the cation. Glucose isomerase may be engineered to increase the association rate for the cation by the addition of negative charge (or deletion of positive charge) near the active site channel, or to decrease the association rate for the cation by the addition of positive charge (or deletion of negative charge) near the active site channel. Since the offrate is not expected to be affected substantially, an altered on-rate will result in an altered overall association constant of the cation. Since the loop regions situated at the C-termini of the β-barrel shape the active site entry, the possible mutation sites are searched in these regions. To avoid possible interference with barrel stability, substitutions in β-strands or α-helices will not be considered. The following rational may be used :

- Select all residues in the region between the C-terminal ends of the β-strands and the N-terminal ends of the α-helices.
- Reject from further consideration all residues where substitution leads to a decrease of the net positive charge in a sphere of 15 Ångstroms radius around the metal ligands. Introduction of negative charges too close to the metal binding side will shift the pK_a of the metal ligands to a higher pH, which will cancel out the effect of increased K_{ass} at low pH.
- Compute for each of the remaining residues its accessible surface area in the context of the protein and

using a probe of radius 1.4 Å. Reject residues that are buried in the sense that they have less than 10 $Å^2$ accessible surface area.

STRUCTURAL INFORMATION

Information on the 3D structure of the enzyme (or enzyme :substrate or enzyme :inhibitor complex) is of great importance to be able to make predictions as to the mutations which can be introduced.

Structural data have been reported for glucose isomerase of Streptomyces rubiginosus (Carrell et al, J. Biol. Chem. 259 (1984) 3230-3236) ; Carrell et al. proc. Natl. Acad. Sci USA 86, (1989) 440-4444) Streptomyces olivochromogenus (Farber et al, Protein Eng. 1, (1987) 467-475 ; Farber et al. Biochemistry 28 (1987) 7289-7297), Arthrobacter (Hendrick et al., J. Mol. Biol. 208 (1989) 127-157) and Streptomyces albus (Dauter et al FEBS Lett. 247, 1-8).

Although not all amino acid sequence data are available for these enzymes the 3D-structural homology with Actinoplanes missouriensis glucose isomerase is striking (see F. Rey et al., Proteins 4 (1988) 165-172). To show the general applicability of the method disclosed in this specification the genes for glucose isomerase originating from various species have been cloned and sequenced. The amino acid sequences of glucose isomerases as deduced from the genes of Streptomyces violaceoruber, Streptomyces murinus, Arthrobac- ter spec. and Streptomyces thermovulgaris are shown to be homologous. Published amino acid sequences for the glucose isomerases of Ampullariella sp. (Saari, ibid.) and Streptomy- ces violaceoniger (Nucl. Acids Res. 16 (1988) 9337), deduced from the nucleotide sequences of the respective genes, display a close homology to Actinoplanes missouriensis glucose isomerase. In addition, WO 89/01520 discloses that the amino acid sequence of Streptomyces rubiginosus glucose isomerase is homologous to Ampullariella sp. glucose isomerase.

Despite the absence of 3D structural data for most glucose isomerases, it can be concluded that all glucose isomerases from Actinomycetales have a similar tetrameric organisation.

In general, it can be assumed that where the overall homology is greater than 65%, preferably greater than 74% (minimal homology between Actinoplanes missouriensis and Streptomyces glucose isomerase, according to Amore and Hollenberg, Nucl. Acids Res. 17, 7515 (1989)), and more preferably greater than 85% and where the 3D structure is similar, amino acid replacements will lead to similar changes in pH optimum. Specifically one expects the glucose isomerases from species belonging to the order of the Actinomycetales to have such a high degree of similarity that the alteration of pH optimum due to amino acid replacements at the selected sites are similar. Actinoplanes missouriensis is the preferred source of glucose isomerase to mutate.

Figure 1 gives a schematic presentation of the active site of the glucose isomerase from Actinoplanes missouriensis.

Figure 2 shows the aligned amino acid sequences of various glucose isomerases.

In the present specification both the three letter and the one letter code for amino acids is used (see e.g. Stryer, L. Biochemistry, p.13, 2nd ed, W.H. Freeman and Comp., NY, 1981).

EXPERIMENTAL

Cloning and expression of the D-glucose isomerase gene

D-glucose isomerase (GI) is synonymously used for D-xylose isomerase ((D-xylose) ketol-isomerase, EC 5.3.1.5), an enzyme that converts D-xylose into D-xylulose. The D-glucose isomerase from Actinoplanes missouriensis produced by engineered E. coli strains is designated as EcoAmi (DSM) GI. To distinguish the Actinoplanes missouriensis gene coding for GI from the analogous E. coli xylA gene, the former will be designated as GI.

Methods for manipulation of DNA molecules are described in Maniatis et al. (1982, Cold Sprong Harbor Laboratory) and Ausubel et al. (1987, Current Protocols in Molecular Biology, John Wiley & Sons Inc. New York). Cloning and DNA sequence determination of the glucose isomerase gene from Actinoplanes missouriensis DSM 43046 is described in EP-A-0351029. The derived amino acid sequence of GI is numbered and compared with other glucose isomerases in Figure 2. In the following, the numbering of amino acids refers to Figure 2.

Wildtype and mutant GI enzymes were produced in E. coli strain K514 grown as described in EP-A-0351029.

Assay of the enzymatic activity of the expression product

The enzymatic activity of glucose isomerase was assayed as described below (1 unit of enzymatic activity

produces 1.0 micromole of product -D-xylulose or D-fructose-per minute ; therefore, specific activity -spa- is expressed as units per mg of GI enzymes).

GI activity can be assayed directly by measuring the increase in absorbance at 278 nm of xylulose produced at 35°C by isomerisation of xylose by glucose isomerases. This assay was performed in 50 mM triethanolamine buffer, pH 7.5, containing 10mM $MgSO_4$, in the presence of 0.1 M xylose. Glucose isomerase final concentration in the assay was ± 0.01 mg/ml, and precisely determined, prior to dilution in the enzymatic assay mixture, by absorption spectroscopy using an extinction coefficient of 1.08 at 278 nm for a solution of enzyme of 1.0 mg/ml.

The specific activity was determined in the D-Sorbitol Dehydrogenase Coupled Assay, enzymatic determination of D-xylulose was performed at 35°C as previously described (Kersters-Hilderson et al., Enzyme Microb. Technol. 9 (1987) 145) in 50mM triethanolamine, pH 7.5, 10mM $MgSO_4$, and 0.1 M xylose, in the presence of ± 2 x 10-8 M D-sorbitol dehydrogenase (L-iditol : NAD oxidoreductase, EC 1.1.14), and 0.15 nM NADH, except that the incubation buffer also included 1mM ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA). Glucose isomerase final concentration in this assay was ± 2.5 x 10^3 mg/ml, and precisely determined as described above.

With glucose as a substrate GI activity can be assayed by the measurement of D-fructose produced during the isomerization reaction using the cysteine-carbazole method (CCM) which is based on the reaction of ketosugars with carbazole in acids to yield a purple product (Dische and Borenfreund, J. Biol. Chem. 192 (1951) 583).

EXAMPLE 1

The pH dependence of glucose isomerase activity

In order to determine the pH-activity profile of wild-type and mutant glucose isomerase, the activity was measured as a function of pH (5.2-8.0) in the presence of 10 mM $MgSO_4$ and 200 mM xylose (using the direct assay method). For mutants with very low activity the coupled sorbitol dehydrogenase assay system was used between pH 5.8 and 8.4. Care was taken that the sorbitol dehydrogenase reaction did not become rate limiting at extreme pH values.

The pH-activity profile of glucose isomerase (i.e. the recombinant wildtype enzyme from Actinoplanus missouriensis) in the presence of 200 mM xylose and different activating cations is shown in Figure 3. It reveals a decrease in activity at both acidic pH below 7.0 and at alkaline pH beyond 8.0.

The appropriate steady-state kinetic mechanism for glucose isomerase involves the rapid formation of an enzyme-metal-sugar complex which is converted to the product in a rate limiting step (so called rapid equilibrium, random ordered mechanism - see Figure 4). Equilibrium and transient kinetic fluorescence measurements (stopped flow) indicate the presence of two metal ion binding sites. In the stopped flow experiment the metal ions bind consecutively. The high affinity metal plays a role in maintaining an active conformation and is therefore called the 'conformational' site. The second metal binding site accommodates the activating cation, therefore this site is usually indicated as the 'catalytic' site. The reaction scheme which is shown in Figure 4 appears to be adequate to analyze and compare steady-state and stopped flow experiments. In principle steady-state results do not distinguish between the two metal binding sites, but it is assumed that the main effect comes from the catalytic metal binding.

Analysis of the initial rate (v) of xylose conversion as a function of the xylose and magnesium concentrations allows to determine four parameters : the maximal velocity, the equilibrium constants for magnesium binding to the free enzyme (K_1), the enzyme-sugar complex (K_4), and the equilibrium constants of xylose binding to the free enzyme (K_2) and the enzyme-magnesium complex (K_3).

$$\frac{V_{max}}{v} = 1 + \frac{1}{K_3*[S]} + \frac{1}{K_4*[M]} + \frac{1}{K_3*K_1*[S]*[M]}$$

where [M] and [S] represent the concentration of the metal ion and xylose respectively.

By systematic variation of the magnesium ion and the xylose concentrations it was possible to obtain values for the maximal velocity and for all four equilibrium constants. Figure 5 shows the pH dependance of these parameters.

Comparison of the results of Figures 3 and 5, shows that the acidic side of the pH profile is completely determined by the metal ion binding.

The data of Figure 5 are not sufficiently accurate to calculate the number of ionizations involved. The slope of

the plots of $\log K_{1,4}$ vs pH (slope > 1) indicates however, that more than one ionization may be involved. The similarity in pH dependence of K_1 and K_4 indicates that the same ionizing groups are important for both these processes (involving the same site).

EXAMPLE 2

Selection of amino acid residues in glucose isomerase of which substitution will alter the pK_a values of the metal binding, ionizable amino acids.

In the case of glucose isomerase, the criteria for the selection of possible positions for substitution, as outlined in the detailed description of this invention, were applied using the aligned sequences from different sources (Figure 2) and the highly refined structure of Actinoplanes missouriensis glucose isomerase in complex with the inhibitor xylitol (see Figure 1 and "Structural Information" in the "Detailed description of the invention"). The highly refined structure with a resolution of 2.2 Ångstroms reveals the position of the inhibitor and two metal binding sites. A schematic representation of the active site of glucose isomerase of Actinoplanes missouriensis is given in Figure 1.

From the glucose isomerase structure complexed with cobalt and xylitol those residues were selected where substitution into a more positively charged amino acid residue leads to a net increase in positive charge within a 15 Angstrom radius around the target ionizable groups. In the case of glucose isomerase the targets are the ionizable goups that are involved in the coordination of the metal ions required for activity. These target ionizable groups imply the carboxylgroups of Glu181, Glu217, Asp245, Asp255, Asp292 and the NE of His220.

After application of criterion I, 80 possible mutation sites were retained. These sites are summarized below: Ala5, Phe11, Leu15, Trp20, Gln21, Ala25, Phe26, Asp28, Ala29, Gly47, Tyr49, Thr52, Phe53, His54, Asp56, Asp57, Phe61, Ile85, Met88, phe94, Thr95, phe104, Gln122, Thr133, Leu134, Val135, Ala143, Tyr145, Tyr158, Asn163, Ser169, Glu181, Asn185, Glu186, Gly189, Ile191, Pro194, His198, Gln204, Leu211, Phe212, Asn215, Glu217, Thr218, His220, Glu221, Gln222, Ser224, Asn225, Leu226, Phe228, Thr229, Gly231, Leu236, His238, His243, Asp245, Asn247, His250, Phe254, Asp255, Gln256, Asp257, Leu258, Val259, Phe260, His262, Leu271, Tyr285, Asp286, His290, Asp292, Tyr293, Thr298, Glu299, Trp305, Ala310, Met314, Val380, Asn383.

After discarding the catalytic residues and the strictly conserved residues (criterion II) the following 62 residues are left :
Ala5, Leu15, Gln21, Ala25, Phe26, Asp28, Ala29, Gly47, Tyr49, Thr52, Asp56, Phe61, Ile85, Met88, Thr95, Phe104, Gln122, Thr133, Leu134, Ala143, Tyr145, Tyr158, Asn163, Ser169, Asn185, Gly189, Ile191, Pro194, His198, Gln204, Leu211, Phe212, Thr218, Gln222, Ser224, Asn225, Leu226, Phe228, Thr229, Gly231, Leu236, His238, His243, His250, Phe254, Gln256, Asp257, Leu258, Val259, His262, Leu271, Tyr285, Asp286, His290, Tyr293, Thr298, Glu299, Trp305, Ala310, Met314, Val380, Asn383.

Subsequently a priority was attributed to each of these 62 possible mutation sites according to criterion III. The following 24 sites were attributed as having high priority for mutagenesis : Ala25, Gly47, Tyr49, Thr52, Phe61, Ile85, Thr95, Gln122, Thr133, Tyr145, Tyr158, Gly189, Ile191, Gln204, Thr218, Gln222, Leu226, Thr229, Gly231, Leu236, Gln256, Leu258, Tyr293 and Val380.

Mutation of either one or several of the 80 selected amino acid residues into positively charged residues will result in a decrease of the pK_a of metal binding of glucose isomerase. This may result in a corresponding shift of the pH-activity profile towards lower pH.

Correspondingly, mutation of either one or several of the 80 selected amino acid residues into negatively charged residues will result in an increase of the pK_a of metal binding of glucose isomerase. This may result in a corresponding shift of the pH-activity profile towards higher pH.

EXAMPLE 3

The effect of mutating Lys 294 on the pH-activity profile

At position 294 in glucose isomerase a positive charge is located about 8 Ångstroms from the highest occupied metal binding site in the glucose isomerase-xylitol complex (395 in Figure 1). A mutation at this site was made, replacing the lysine for an arginine. This mutation conserves the positive charge at position 294. Consistent with this conservation of the positive charge is the observation that the pH-activity profile of this mutant is similar to that of the wildtype enzyme.

However, when at position 294 the positive charge is removed by replacing lysine 294 by a glutamine we observed a shift of the pH-activity profile towards the alkaline site by approximately 0.5 pH units. The pH-activity profiles for K294R and K294Q are shown in Figure 6.

This example illustrates that it is possible to manipulate the pK_a of one or more functional groups, in the active site of glucose isomerase, by changing the net charge of the protein around the active site.

EXAMPLE 4

The effect of mutating Glu186 and replacing magnesium ions by manganese ions on the pH-activity profile

In the mutant E186Q a negative charge is replaced by a neutral one which gives rise to a net increase in positive charge within a 15 Ångstroms radius around the metal ligands. The pH-activity profile of the E186Q mutant in the presence of magnesium is shown in Figure 7. The alkaline flank of the pH profile is shifted significantly toward lower pH. In the presence of manganese instead of magnesium the pH-activity profile of E186Q is shifted to a lower pH and at its optimum pH its activity is higher than for the wildtype (Figure 8).

For applications where metal ions other than magnesium can be used the combination E186Q with manganese at low pH is an interesting option.

We also performed the mutation E186D which is conservative with respect to charge. The pH-activity profile of this mutant is shown in Figures 7 and 8. The pH dependence of the activity for the E186D mutant is not significantly different from that of the wildtype enzyme. Removal of the negative charge at position 186 did shift the pH activity profile to a more acidic pH. This example emphasizes that the rationale of the mutation E186Q holds.

EXAMPLE 5

The effect of replacing Asp255 on the pH-activity profile

Substitution of the negatively charged aspartic acid at position 255, which is in fact a metal binding ligand in glucose isomerase, by a neutral asparagine, gives rise to a shift of the pH-activity profile towards lower pH in the presence of manganese. The pH optimum shifts about 2 pH units towards more acidic pH.

The pH-activity profile is given in Figure 9.

EXAMPLE 6

Glucose isomerases with an altered pH-activity profile

Mutants of glucose isomerase which were created according to the methods as outlined in the detailed description of the invention, were tested for their pH-activity relation under conditions which are indicated in the Figures (10-20). The pH-activity profile of a mutant is the result of the effect of the mutation on the pK_a on the one hand, and the effect on the K_{ass} on the other hand.

The results for the following mutants are given in the Figures :

F254K (Fig.10), F94R (Fig.11), F61K (Fig.12), A25K (Fig.13), D57N (Fig.14), L258K (Fig.15), Q204K (Fig.16), R23Q (Fig.17), H54N (Fig.18), H290N (Fig.19), T95D (Fig.20).

For the mutants in which a positive charge (F254K, F94R, F61K, A25K, L258K, Q204K) was introduced or a negative charge neutralized (D57N), it can be seen that the acidic-side of the pH-activity profile is shifted towards lower pH.

For mutants in which a negative charge was introduced (T95D) or a positive charge was neutralized (R23Q, H54N, H290N, it can be seen that the pH-activity profile is shifted towards higher pH.

Both of these observations are in agreement with the model as presented in the detailed description of the invention.

However, it should be noted that mutants in which a conserved amino acid has been replaced (F94R, D57N, H54N) give a drastic decrease in specific activity on xylose. At position 254 in the sequence alignment (Fig. 2) only hydrophobic amino acids are found. Introduction of a charged amino acid (F254K) at this position also leads to a drastic decrease in specific activity. Thus, it can be concluded that although (semi)conserved amino acid positions can be used to alter charges in order to modify the pH-activity profile they are not preferred sites.

EXAMPLE 7

Stabilization of mutants with an altered pH optimum to obtain better performance under application conditions

The mutants H290N and F61K give the expected shift in the pH-activity profile as described in Example 6. Of these mutants H290N was immobilized as described in EP-A-351029 (Example 7). Application testing of the wildtype and this mutant glucose isomerase was performed as described in the same application (Example 8). The stability is indicated by the first order decay constant (K_d, the lower the decay constant the more stable the enzyme). Table 2 gives the K_d values for the wild-type and mutant glucose isomerases.

Table 2

Decay constants for wildtype and mutant glucose isomerase, immobilized on Lewatit

	K_d (x 10^6 sec^{-1})
Wildtype	2.5
H290N	3.1
K253R	0.7
H290NK253R	1.6
F61KK253R	1.4

As can be seen in Table 2, H290N is destabilized as compared with the wildtype glucose isomerase. K253R was found to stabilize the wildtype glucose isomerase by a factor larger than three. The pH-activity profile of the K253R mutant is identical with that of the wildtype enzyme.
The combination of the pH mutant H290N with the stability mutant K253R shows that pH mutants can be stabilized by introducing mutations that have been shown to stabilize the wildtype enzyme.

In addition Table 2 shows that pH mutant F61K is stabilized with respect to the wiltype enzyme after introduction of K253R.

The acidic shift of the pH-activity profile of F61K is maintained in the double mutant (Fig. 21). This shows that mutations which improve different properties of an enzyme can be combined in a new mutant which harbours the improved properties of the individual mutations (an improved pH optimum and an improved stability in this case).

It is to be understood that the above mentioned examples are meant to demonstrate the concept of the invention and that they are not meant to limit the scope. In view of this it should be clear that combinations of the above mentioned mutations with other mutations leading to altered characteristics, e.g. thermostability, metal binding or substrate specificity, are also encompassed by the subject invention.

Claims

1. A mutant metalloenzyme, obtained by the expression of a gene encoding said metalloenzyme, differing in at least one amino acid from the wildtype enzyme, and characterized in that it exhibits an altered pH-activity profile.

2. A mutant metalloenzyme according to Claim 1, characterized in that the altered pH-activity profile or at least the acidic part thereof is shifted towards a lower pH value.

3. A mutant metalloenzyme according to Claim 1 or 2, characterized in that the substituted amino acid residue is replaced by a more positively charged residue.

4. A mutant metalloenzyme according to any one of Claims 1 to 3, characterized in that the metalloenzyme is glucose isomerase.

5. A mutant glucose isomerase according to Claim 4, wherein the glucose isomerase is obtained from a microorganisms of the order Actinomycetales.

6. A mutant glucose isomerase according to Claim 5, wherein the glucose isomerase is obtained from Actinoplanes missouriensis.

7. A mutant glucose isomerase according to Claim 4 to 6, characterized in that the pH-activity profile or at least the acidic part thereof is shifted towards a lower pH value.

8. A mutant glucose isomerase according to Claim 4 to 7, characterized by having an amino acid substitution within a sphere of 15 Å around the target residues.

9. A mutant glucose isomerase according to any one of the Claims 4 to 8, wherein the substituted amino acid residue is replaced by a more positively charged residue.

10. A mutant glucose isomerase according to any one of the Claims 4 to 9, wherein at least one of the following amino acids, or an amino acid at a corresponding position in an homologous glucose isomerase, is replaced by an amino acid not found in the wildtype enzyme :
Ala5, Phe11, Leu15, Trp20, Gln21, Ala25, Phe26, Asp28, Ala29, Gly47, Tyr49, Thr52, Phe53, His54, Asp56, Asp57, Phe61, Ile85, Met88, Phe94, Thr95, Phe104, Gln122, Thr133, Leu134, Val135, Ala143, Tyr145, Tyr158, Asn163, Ser169, Glu181, Asn185, Glu186, Gly189, Ile191, Pro194, His198, Gln204, Leu211, Phe212, Asn215, Glu217, Thr218, His220, Glu221, Gln222, Ser224, Asn225, Leu226, Phe228, Thr229, Gly231, Leu236, His238, His243, Asp245, Asn247, His250, Phe254, Asp255, Gln256, Asp257, Leu258, Val259, Phe260, His262, Leu271, Tyr285, Asp286, His290, Asp292, Tyr293, Thr298, Glu299, Trp305, Ala310, Met314, Val380, Asn383.

11. A mutant glucose isomerase according to any one of the Claims 4 to 9, wherein at least one of the following amino acids, or an amino acid at a corresponding position in an homologous glucose isomerase, is replaced by an amino acid not found in the wildtype enzyme :
Ala5, Leu15, Gln21, Ala25, Phe26, Asp28, Ala29, Gly47, Tyr49, Thr52, Asp56, Phe61, Ile85, Met88, Thr95, Phe104, Gln122, Thr133, Leu134, Ala143, Tyr145, Tyr158, Asn163, Ser169, Asn185, Gly189, Ile191, Pro194, His198, Gln204, Leu211, Phe212, Thr218, Gln222, Ser224, Asn225, Leu226, Phe228, Thr229, Gly231, Leu236, His238, His243, His250, Phe254, Gln256, Asp257, Leu258, Val259, His262, Leu271, Tyr285, Asp286, His290, Tyr293, Thr298, Glu299, Trp305, Ala310, Met314, Val380, Asn383.

12. A mutant glucose isomerase according to any one of the Claims 4 to 9, wherein at least one of the following amino acids, or an amino acid at a corresponding position in an homologous glucose isomerase, is replaced by an amino acid not found in the wildtype enzyme :
Ala25, Gly47, Tyr49, Thr52, Phe61, Ile85, Thr95, Gln122, Thr133, Tyr145, Tyr158, Gly189, Ile191, Gln204, Thr218, Gln222, Leu226, Thr229, Gly231, Leu236, Gln256, Leu258, Tyr293 and Val380.

13. A mutant glucose isomerase according to Claim 6, characterized in that it contains at least one of the following amino acid replacements :
R23Q, A25K, D57N, H54N, F61K, F94R, T95D, E186D, E186Q, Q204K, K253R, F254K, D255N, L258K, H290N, K294R, K294Q

14. A method for altering the pH-activity profile of glucose isomerase, comprising the substitution of at least one amino acid by an amino acid having a differently charged side chain.

15. A method according to Claim 14, comprising the replacement of an amino acid within a sphere of 15 Å around the target residues, by a more positively charged amino acid.

16. A process for obtaining a mutant glucose isomerase molecule according to any one of claims 1-13 comprising the following steps :
a) obtaining a DNA sequence encoding a glucose isomerase,
b) mutating this sequence at selected nucleotide positions,
c) cloning the mutated sequence into an expression vector in such a way that the DNA sequence can be expressed,
d) transforming a host organism or cell with said vector,
e) culturing said host organism,
f) isolation the glucose isomerase.

17. Use of a mutant glucose isomerase according to any one of the preceding claims in the conversion of sugar molecules.

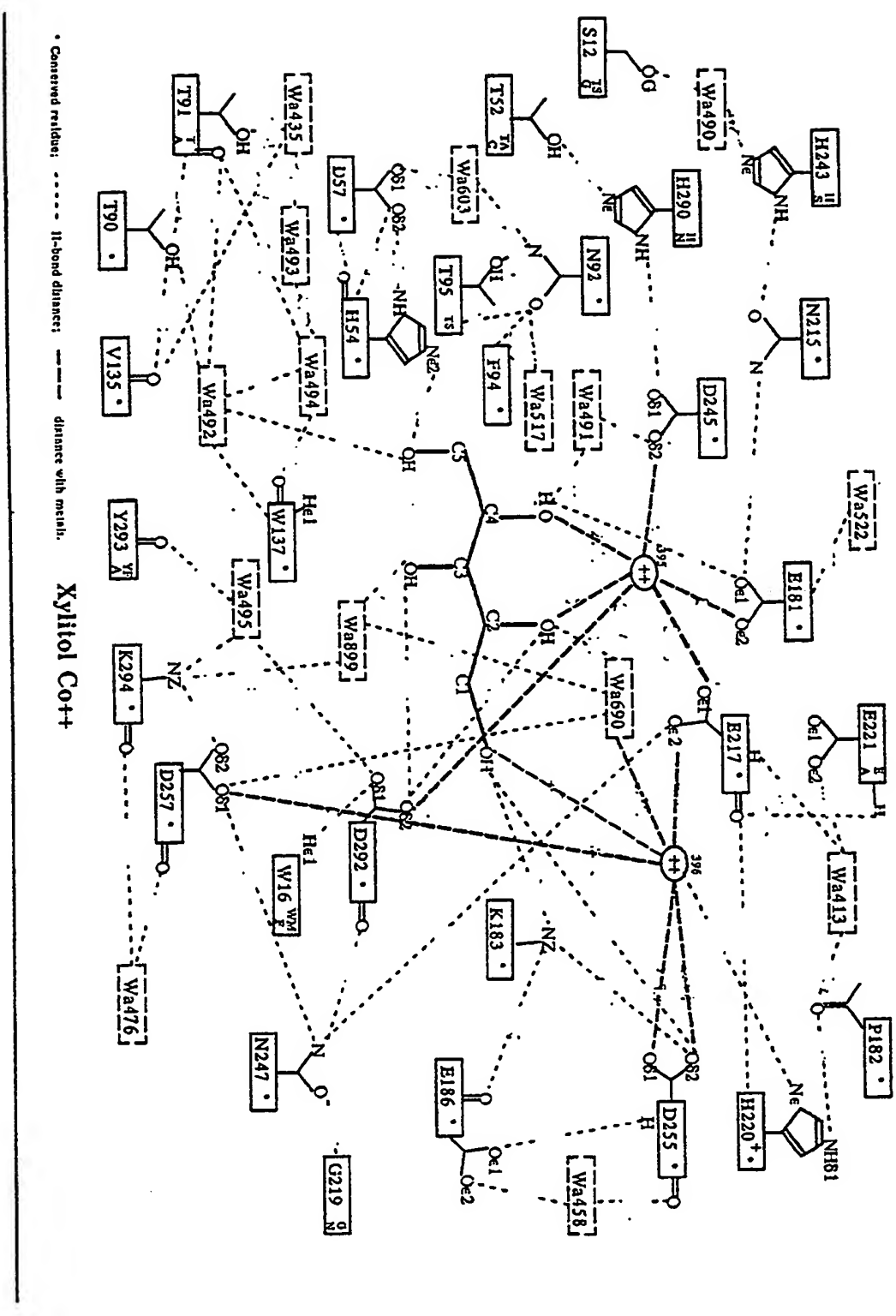
Xylitol Co++
* Conserved residue; ----- H-bond distance; —— distance with metal.

FIGURE 1

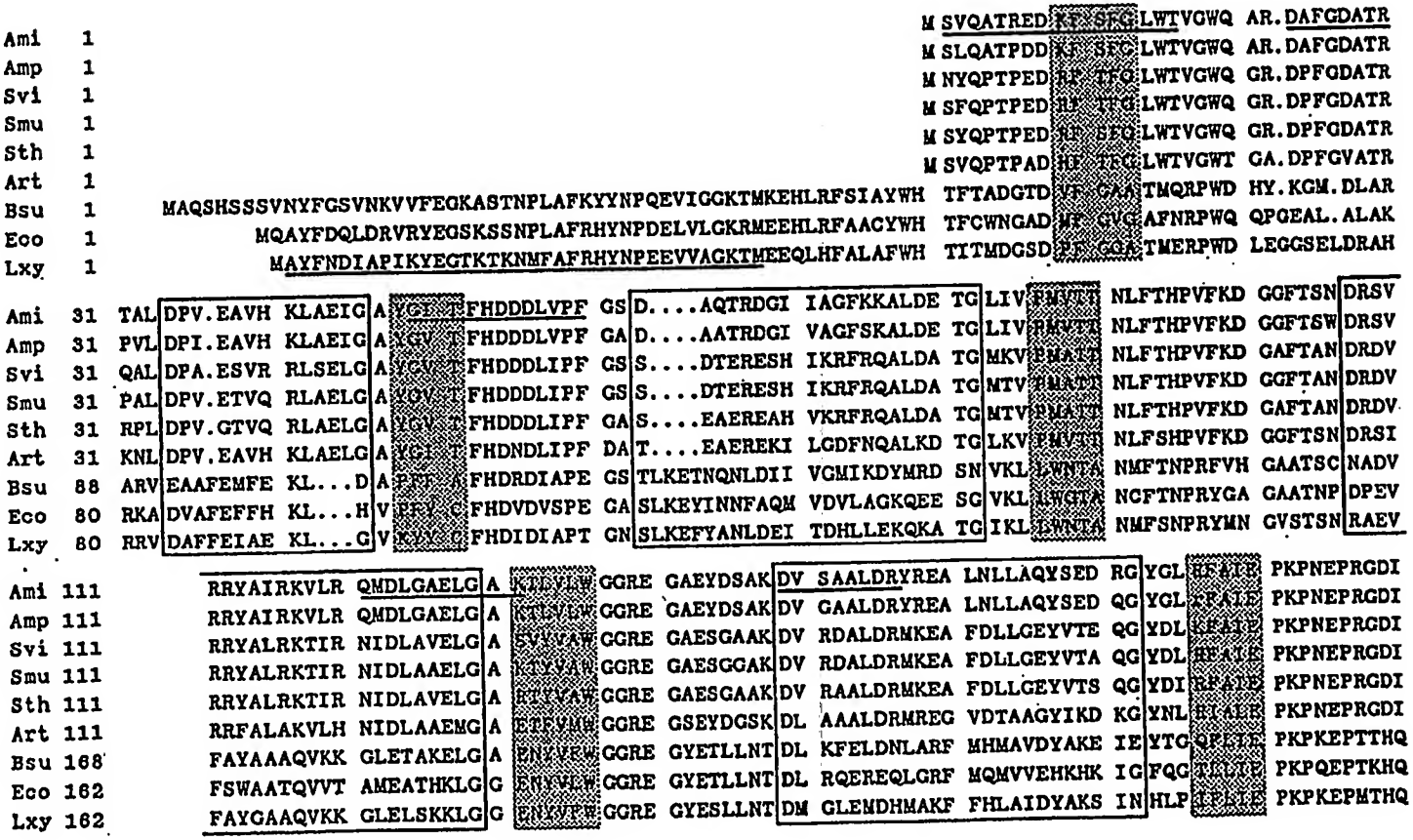

FIGURE 2A

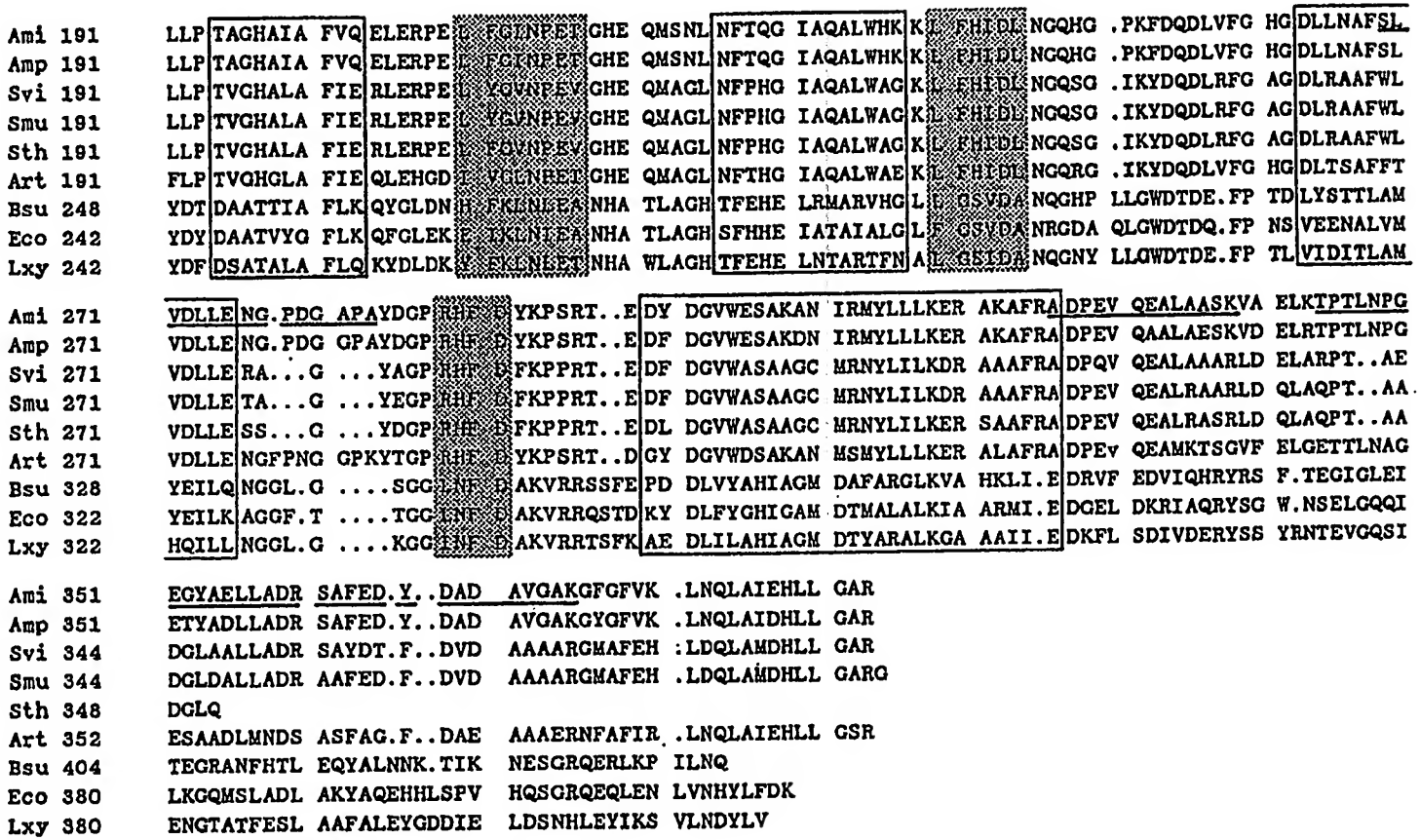

FIGURE 2B

EP 0 436 502 A2

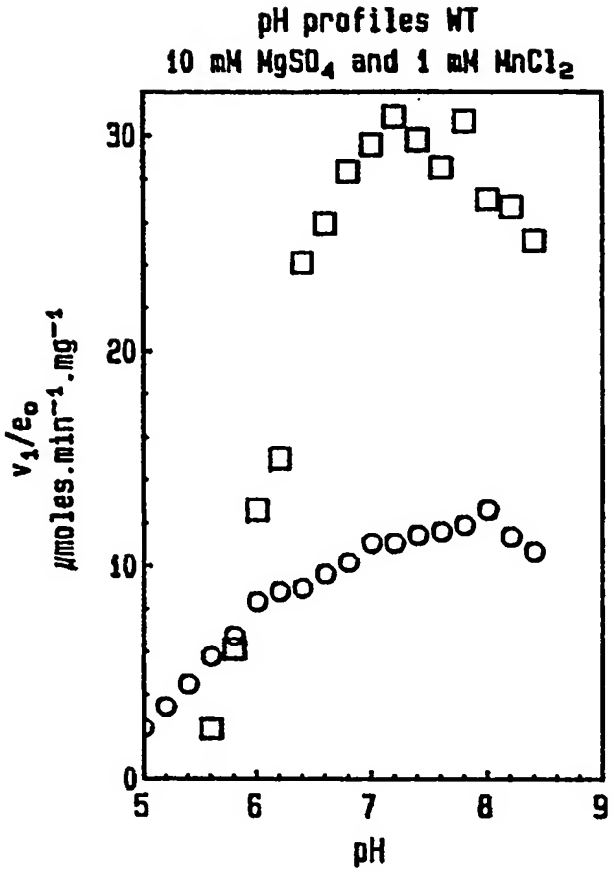
pH profiles WT
10 mM MgSO4 and 1 mM MnCl2
v1/e0
μmoles.min-1.mg-1
0
10
20
30
5
6
7
8
9
pH

FIGURE 3

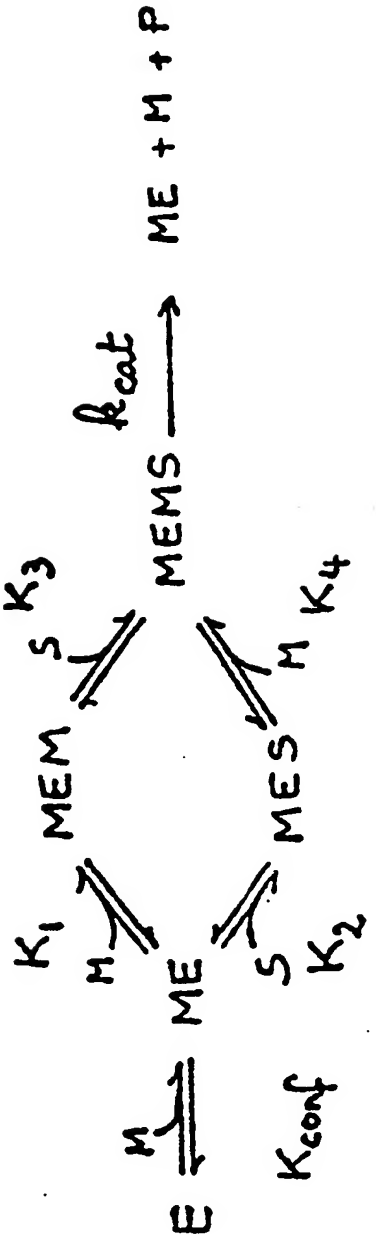

FIGURE 4

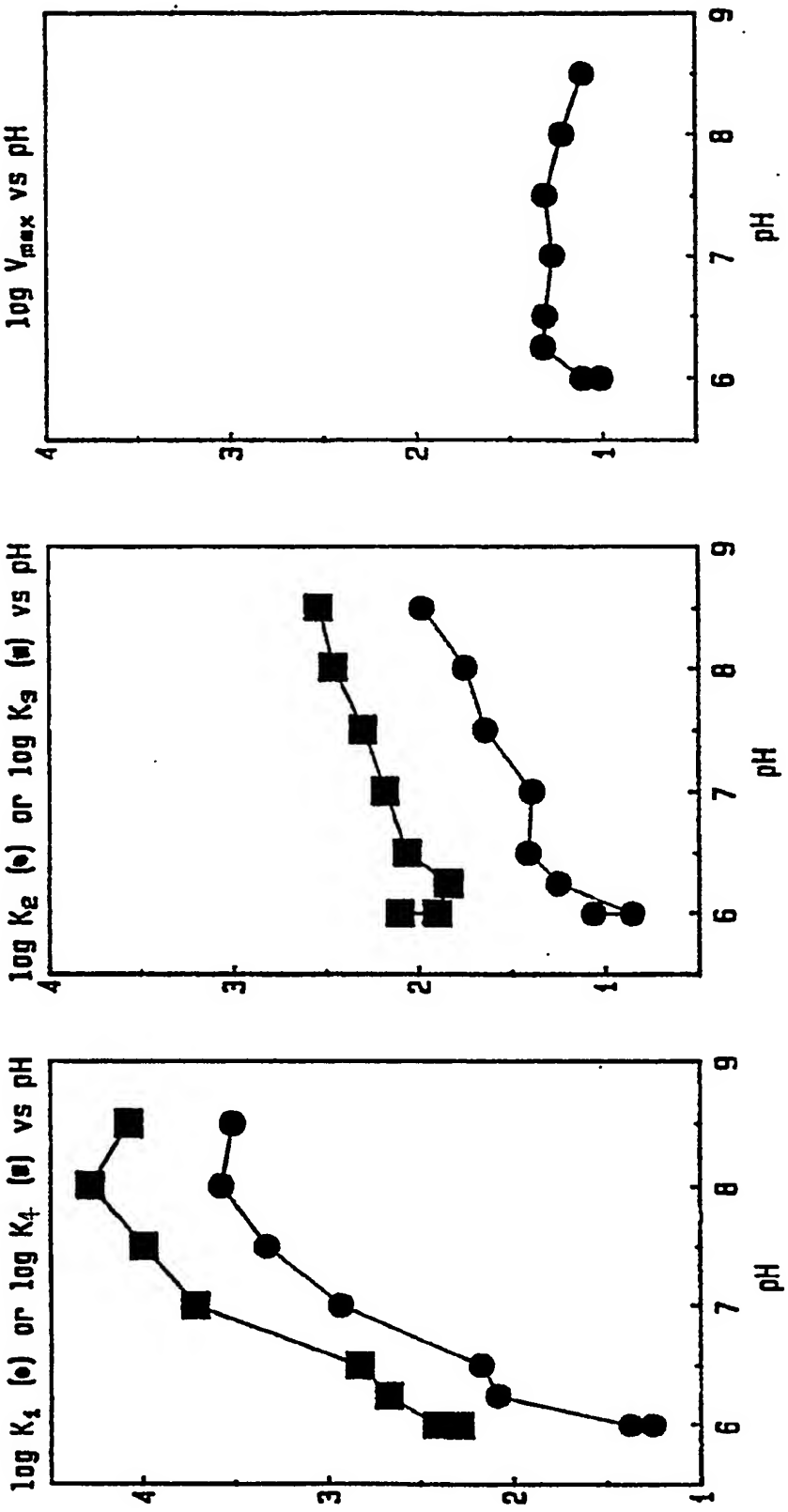

FIGURE 5

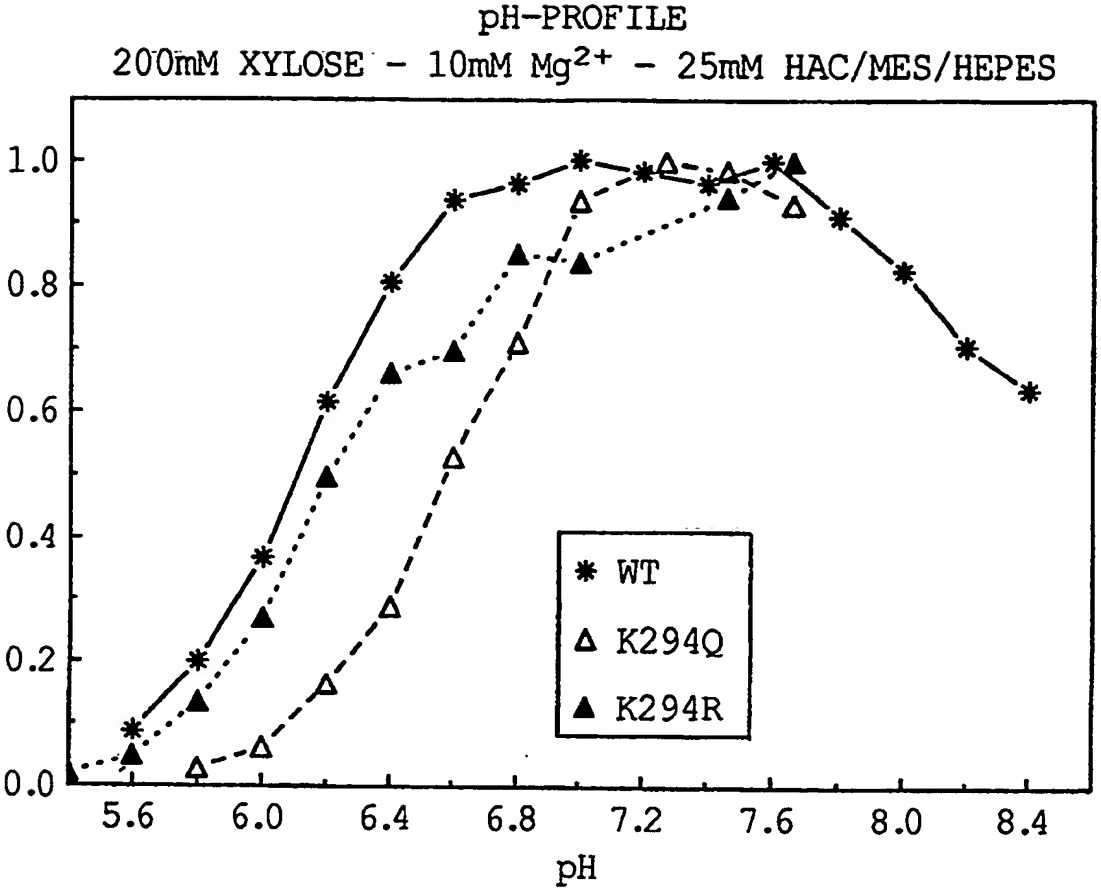
pH-PROFILE
200mM XYLOSE - 10mM Mg2+ - 25mM HAC/MES/HEPES
1.0
0.8
0.6
0.4
0.2
0.0
5.6
6.0
6.4
6.8
7.2
7.6
8.0
8.4
pH
WT
K294Q
K294R

FIGURE 6

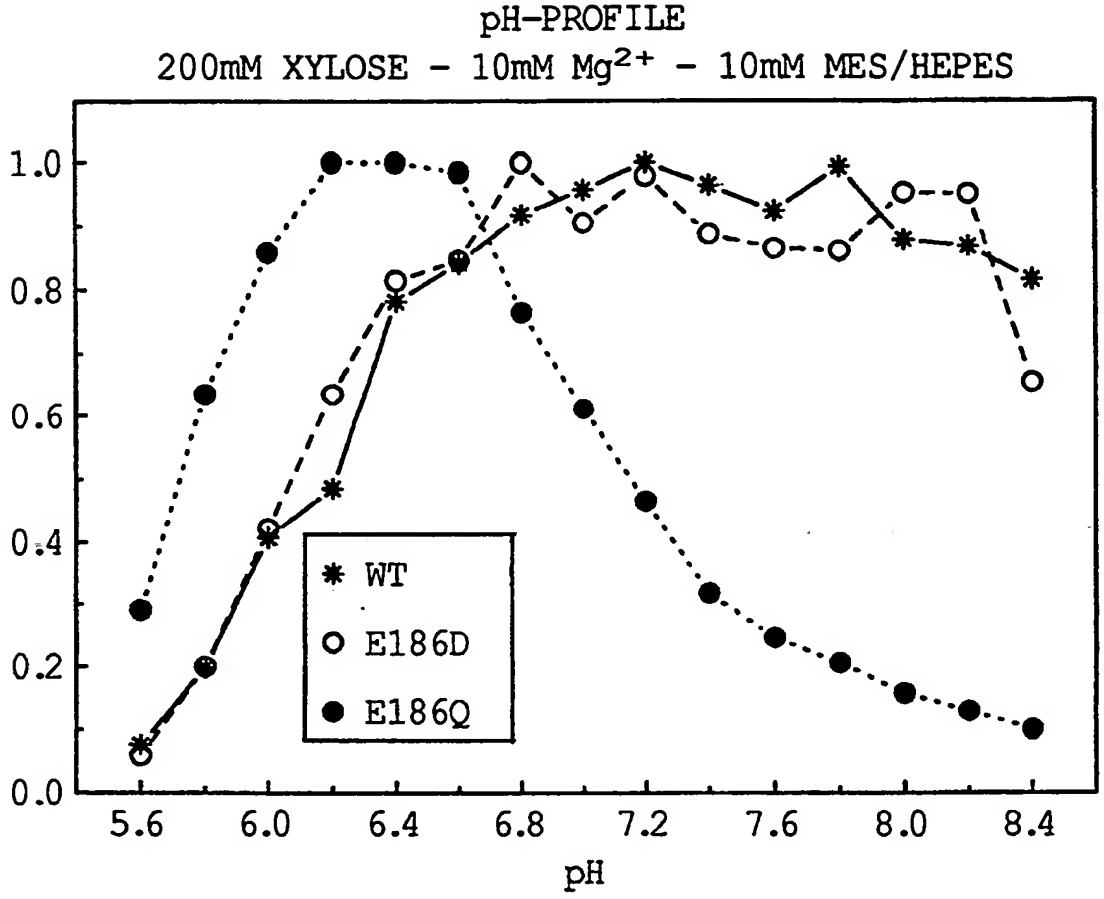
pH-PROFILE
200mM XYLOSE - 10mM Mg2+ - 10mM MES/HEPES
1.0
0.8
0.6
0.4
0.2
0.0
5.6
6.0
6.4
6.8
7.2
7.6
8.0
8.4
pH
WT
E186D
E186Q

FIGURE 7

pH-PROFILE
200mM XYLOSE - 1mM Mn^{2+}- 10 mM MES/HEPES

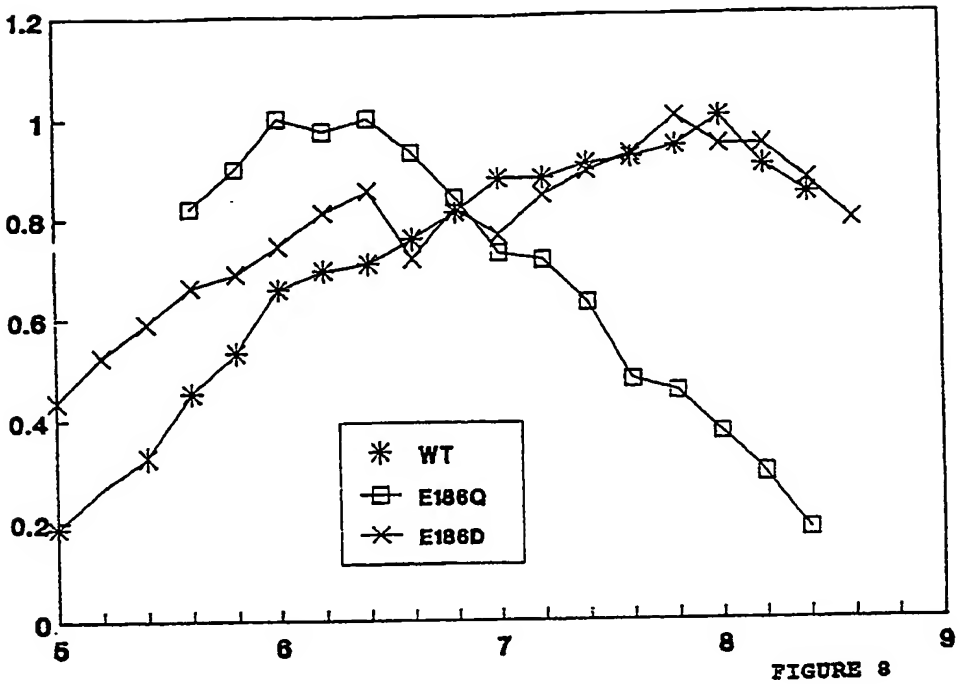

FIGURE 8

pH-PROFILE
200mM XYLOSE - 1mM Mn^{2+}

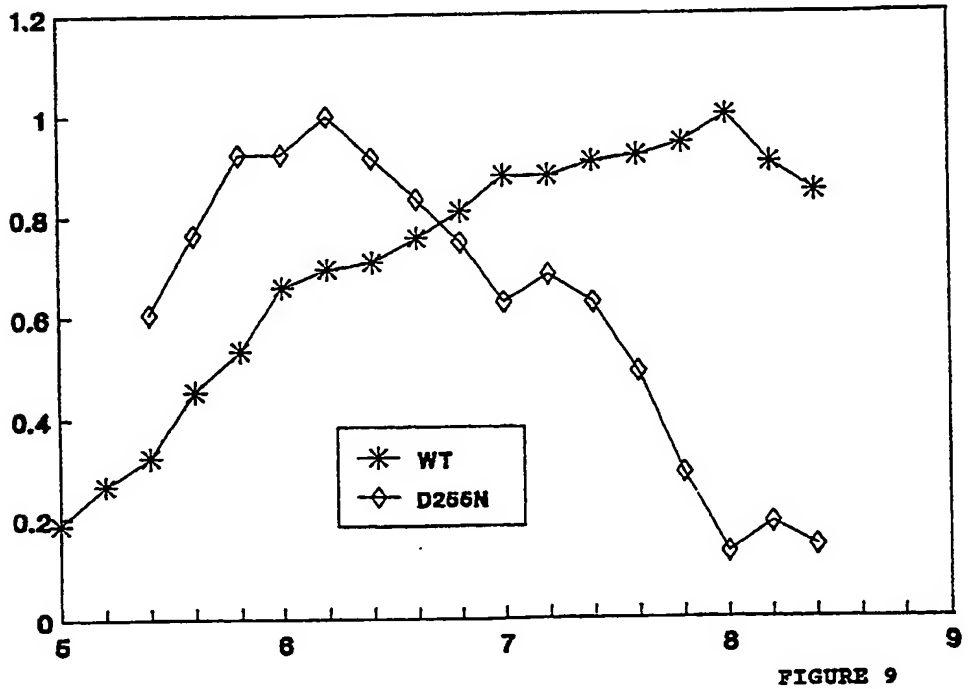

FIGURE 9

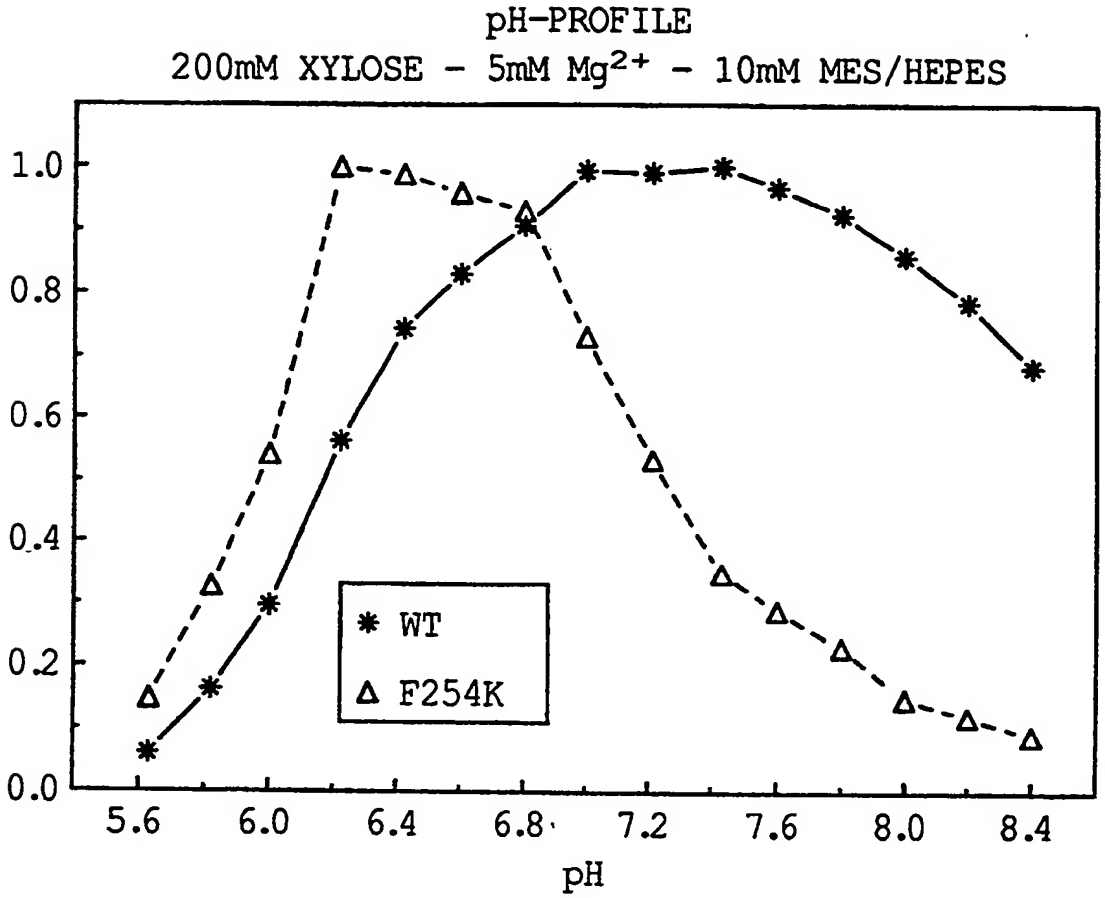
pH-PROFILE
200mM XYLOSE - 5mM Mg2+ - 10mM MES/HEPES
1.0
0.8
0.6
0.4
0.2
0.0
5.6
6.0
6.4
6.8
7.2
7.6
8.0
8.4
pH
WT
F254K

FIGURE 10

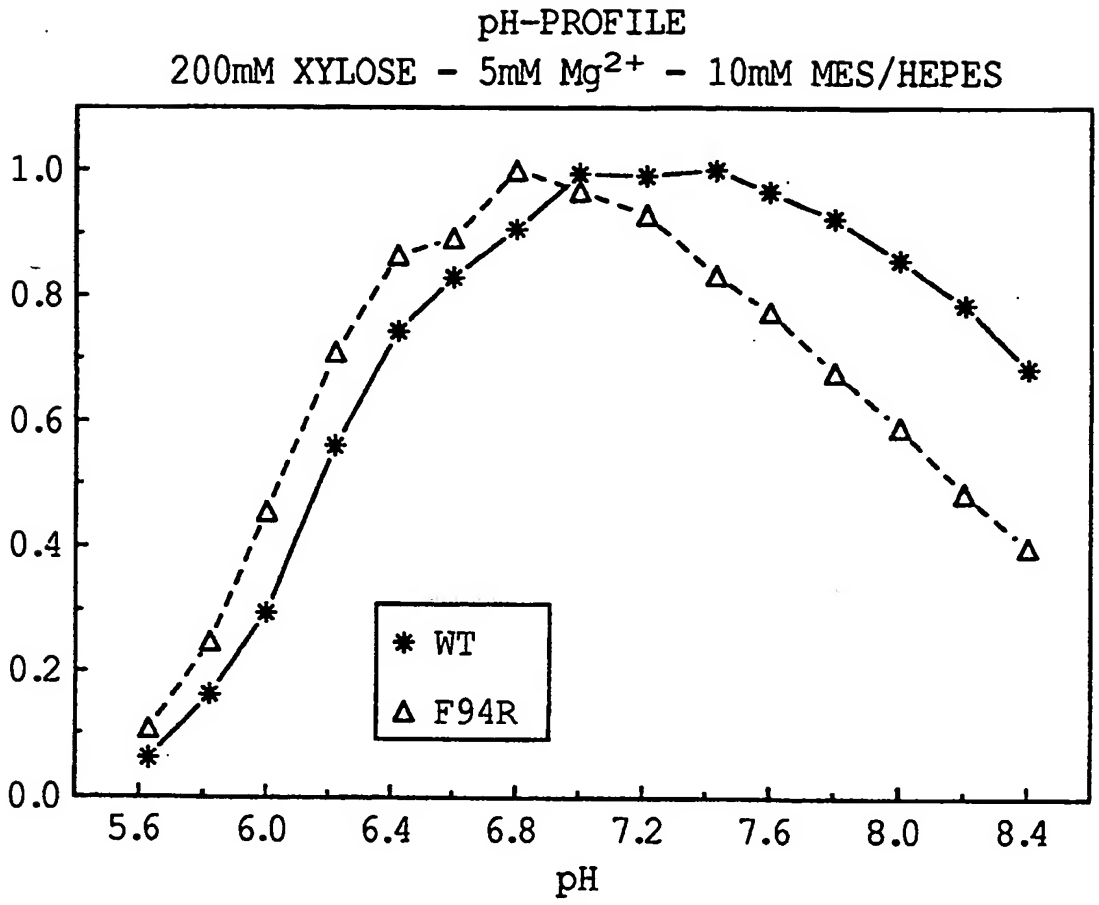
pH-PROFILE
200mM XYLOSE - 5mM Mg2+ - 10mM MES/HEPES
1.0
0.8
0.6
0.4
0.2
0.0
5.6
6.0
6.4
6.8
7.2
7.6
8.0
8.4
pH
WT
F94R

FIGURE 11

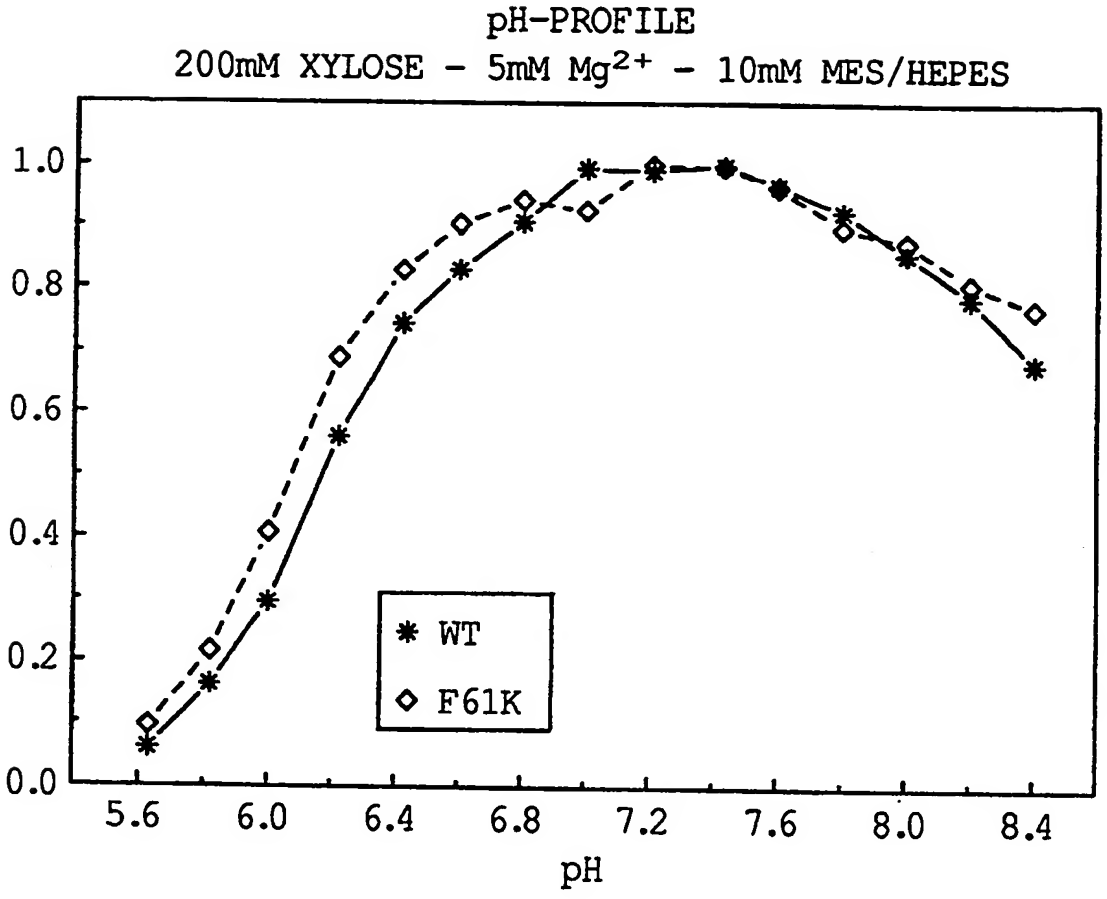
pH-PROFILE
200mM XYLOSE - 5mM Mg2+ - 10mM MES/HEPES
1.0
0.8
0.6
0.4
0.2
0.0
5.6
6.0
6.4
6.8
7.2
7.6
8.0
8.4
pH
WT
F61K

FIGURE 12

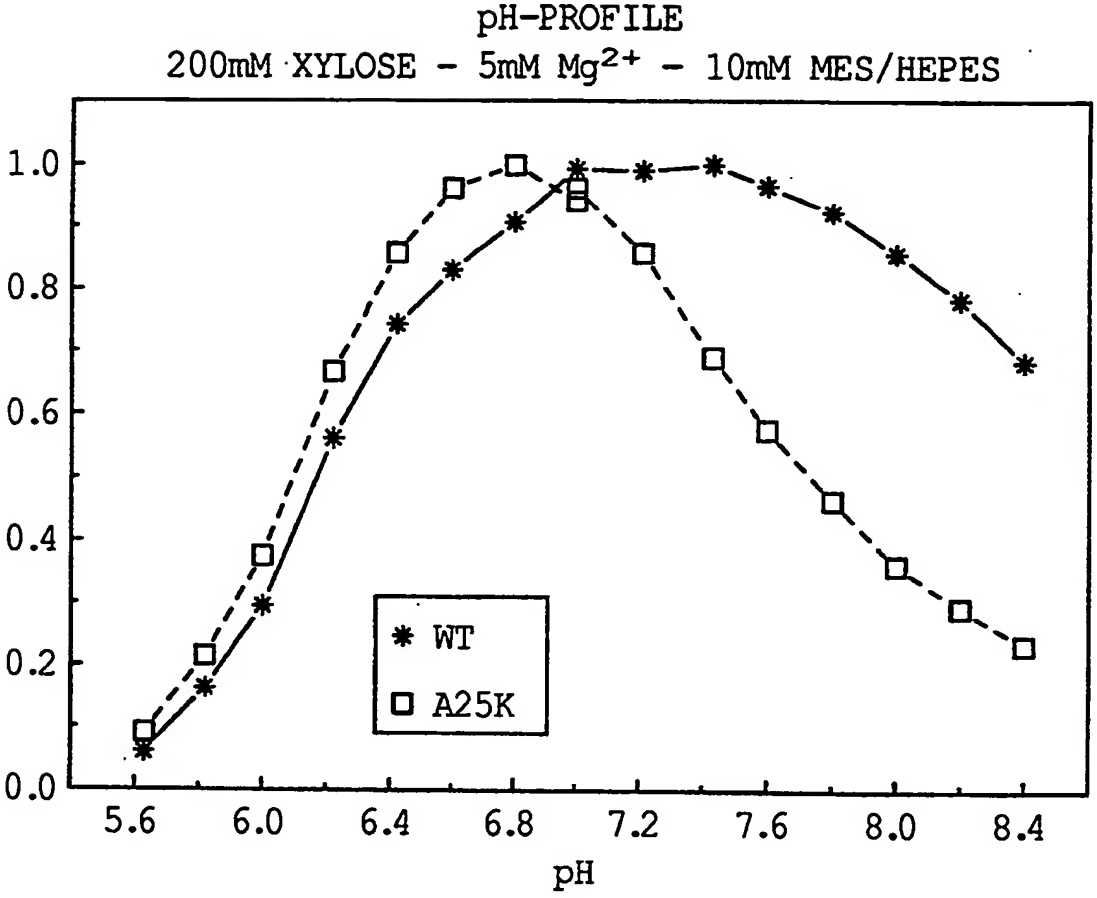
pH-PROFILE
200mM XYLOSE - 5mM Mg2+ - 10mM MES/HEPES
1.0
0.8
0.6
0.4
0.2
0.0
5.6
6.0
6.4
6.8
7.2
7.6
8.0
8.4
pH
WT
A25K

FIGURE 13

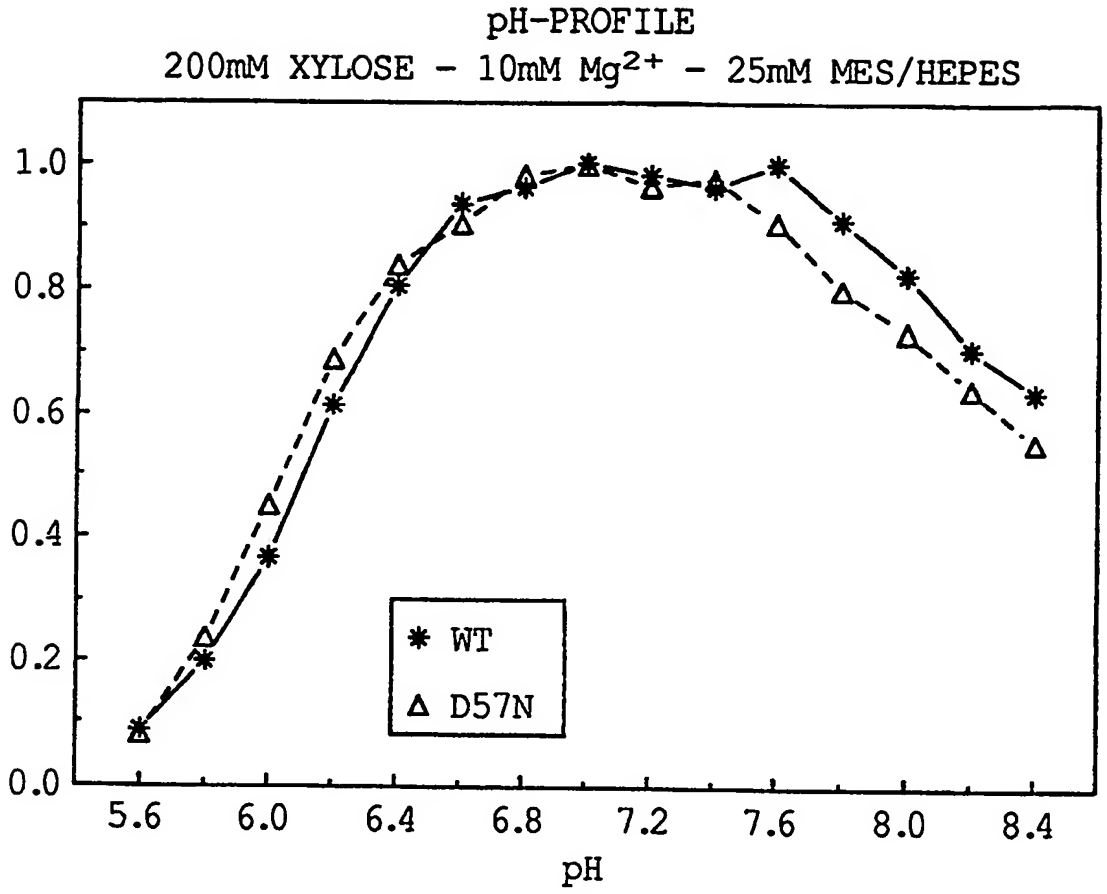
pH-PROFILE
200mM XYLOSE - 10mM Mg2+ - 25mM MES/HEPES
1.0
0.8
0.6
0.4
0.2
0.0
5.6
6.0
6.4
6.8
7.2
7.6
8.0
8.4
pH
WT
D57N

FIGURE 14

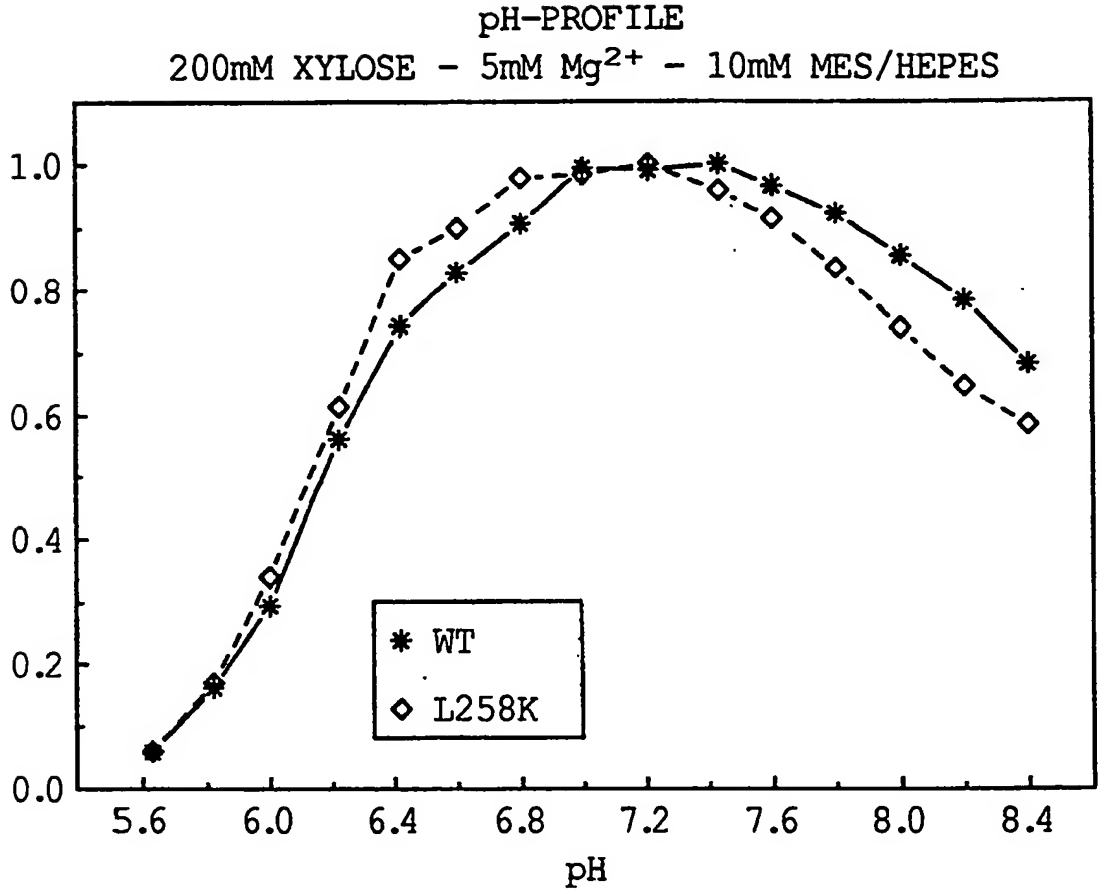
pH-PROFILE
200mM XYLOSE - 5mM Mg2+ - 10mM MES/HEPES
1.0
0.8
0.6
0.4
0.2
0.0
5.6
6.0
6.4
6.8
7.2
7.6
8.0
8.4
pH
WT
L258K

FIGURE 15

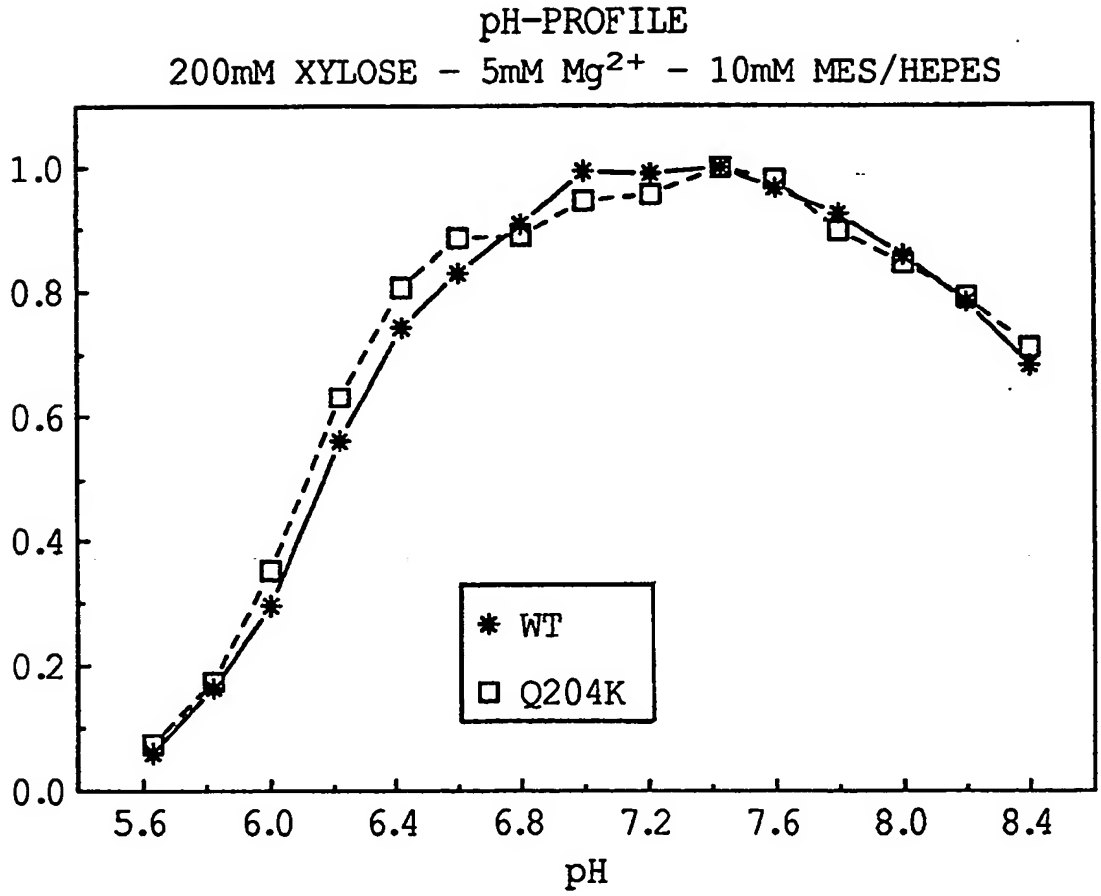
pH-PROFILE
200mM XYLOSE - 5mM Mg2+ - 10mM MES/HEPES
1.0
0.8
0.6
0.4
0.2
0.0
5.6
6.0
6.4
6.8
7.2
7.6
8.0
8.4
pH
WT
Q204K

FIGURE 16

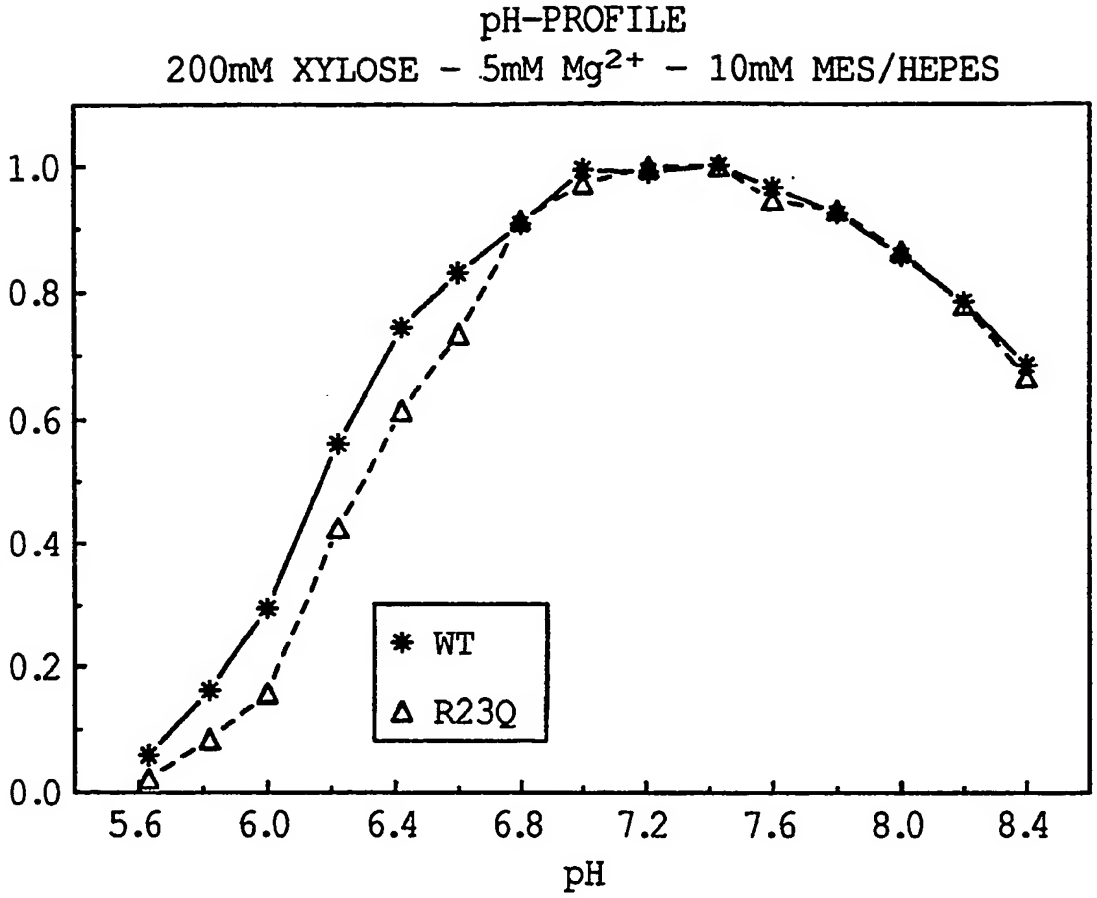
pH-PROFILE
200mM XYLOSE - 5mM Mg2+ - 10mM MES/HEPES
1.0
0.8
0.6
0.4
0.2
0.0
5.6
6.0
6.4
6.8
7.2
7.6
8.0
8.4
pH
WT
R23Q

FIGURE 17

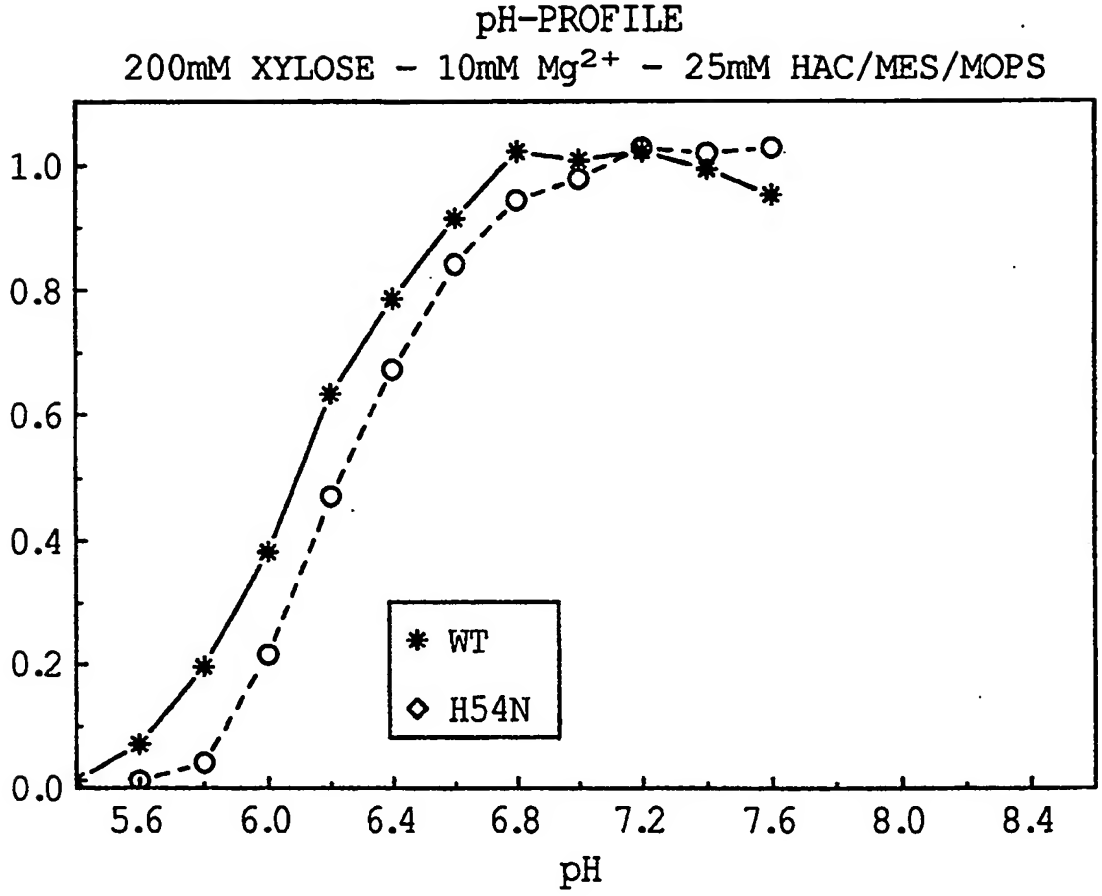
pH-PROFILE
200mM XYLOSE - 10mM Mg2+ - 25mM HAC/MES/MOPS
1.0
0.8
0.6
0.4
0.2
0.0
5.6
6.0
6.4
6.8
7.2
7.6
8.0
8.4
pH
WT
H54N

FIGURE 18

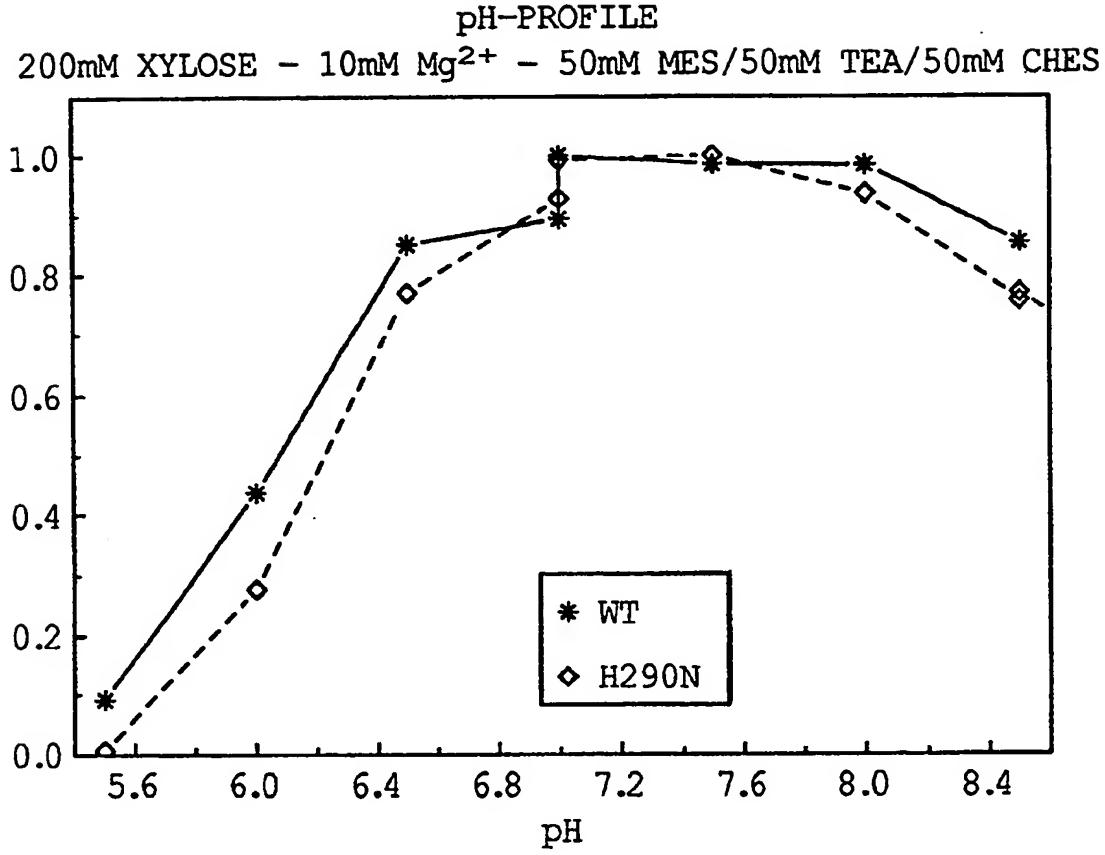
pH-PROFILE
200mM XYLOSE - 10mM Mg^{2+} - 50mM MES/50mM TEA/50mM CHES
1.0
0.8
0.6
0.4
0.2
0.0
5.6
6.0
6.4
6.8
7.2
7.6
8.0
8.4
pH
WT
H290N

FIGURE 19

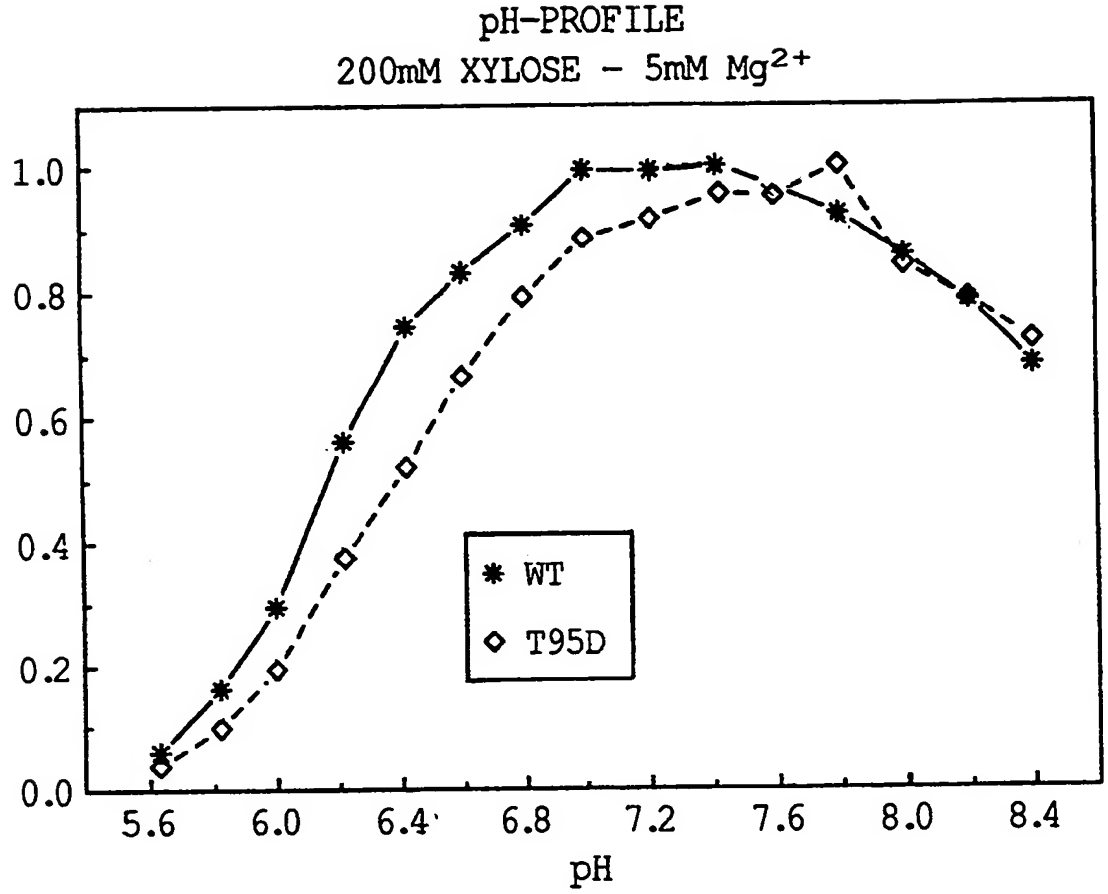
pH-PROFILE
200mM XYLOSE - 5mM Mg^{2+}
1.0
0.8
0.6
0.4
0.2
0.0
5.6
6.0
6.4
6.8
7.2
7.6
8.0
8.4
pH
WT
T95D

FIGURE 20

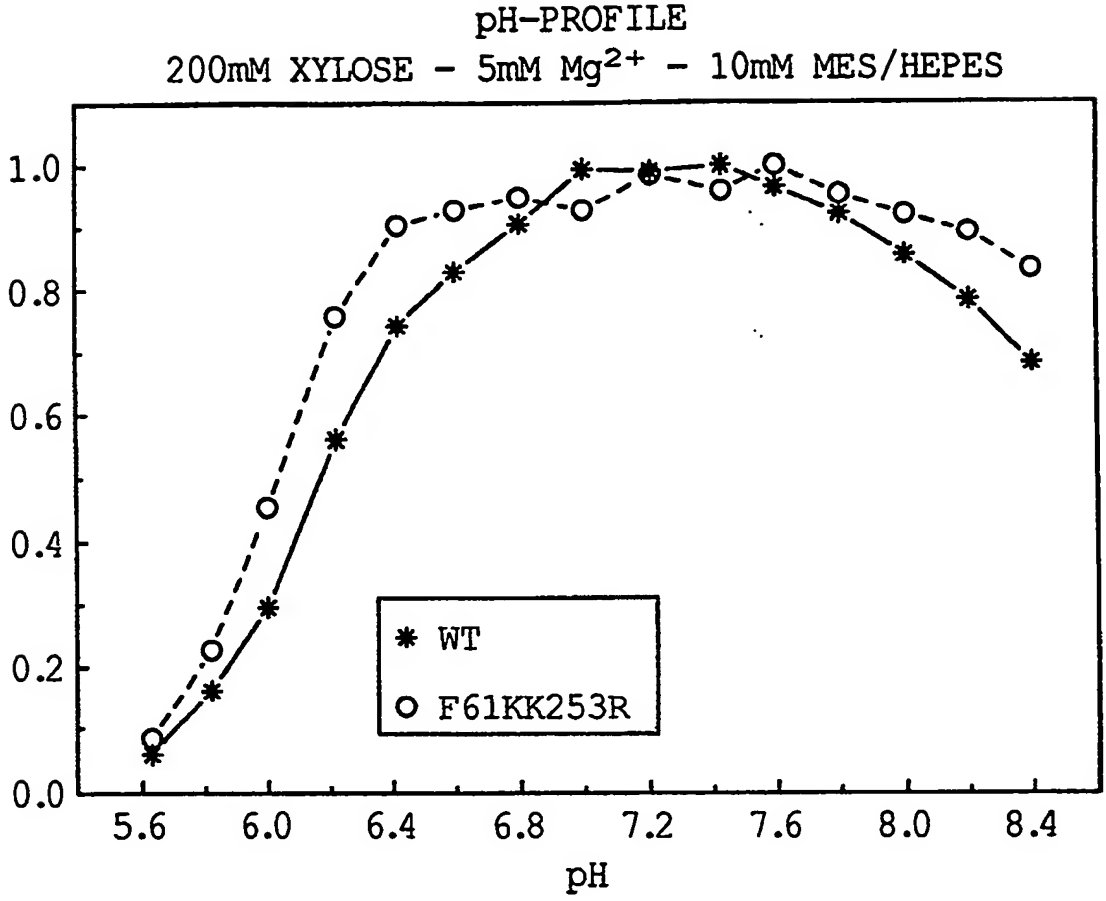
pH-PROFILE
200mM XYLOSE - 5mM Mg2+ - 10mM MES/HEPES
1.0
0.8
0.6
0.4
0.2
0.0
5.6
6.0
6.4
6.8
7.2
7.6
8.0
8.4
pH
WT
F61KK253R

FIGURE 21

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